WATER
WATER
WATER
WATER
WATER
WATER
WATER
WATER
WATER
WATER

PROJECT COMPLETION
REPORT NO. 548

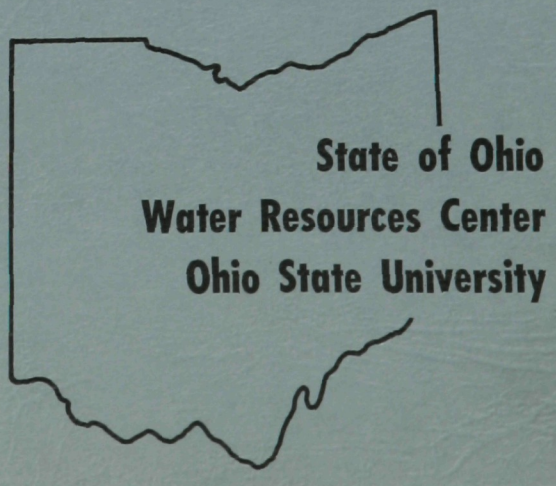
BACTERIAL CONTROL OF AQUATIC ALGAL POPULATIONS

Jeffrey C. Burnham, Ph.D.
Medical College of Ohio

Medical College of Ohio
Toledo, Ohio

United States Department
of the Interior

CONTRACT NO.
B-051-OHIO



State of Ohio
Water Resources Center
Ohio State University

BACTERIAL CONTROL OF AQUATIC ALGAL POPULATIONS

by

Jeffrey C. Burnham, Ph.D.
Medical College of Ohio
Toledo, Ohio 43699

WATER RESOURCES CENTER
THE OHIO STATE UNIVERSITY
Columbus, Ohio 43210

July, 1977

This study was supported in part by the
Office of Water Research and Technology
U. S. Department of the Interior
under
Project B-051-OHIO

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

a) This project was primarily supported by Matching Grant B-051-Ohio from the Office of Water Research and Technology, U. S. Department of Interior. Appreciation is expressed to Dr. Robert C. Stiefel, Director, and the Ohio Water Resources Center, located at Ohio State University, for their administration of this grant program.

b) Acknowledgement is also given to the Medical College of Ohio, and particularly Dr. Russell Claybrook, Associate Dean for Research, and Mr. Oscar Retterer, Grants Administrator, for coordinating administrative and financial assistance for this research program.

c) This project was also supported in part by the Toledo Edison Corporation, Toledo, Ohio, for fiscal years 1974, 1975, 1976, and a General Research Support Grant from the National Institutes of Health (5-501-RR05700-02) for fiscal year 1974.

d) The author wishes to thank Dr. Earl H. Freimer, Chairman of the Department of Microbiology, and the faculty and staff of the Department of Microbiology, for encouraging and stimulating this research project.

e) Appreciation is expressed to Mr. Tom Stetak, Mr. Greg Locher, Mrs. Barbara Highison and Dr. Donald Sun for their assistance in carrying out the research presented in this completed report.

INTRODUCTION

This research project has been a natural evolution from my research experience in the past on bdellovibrios, bacterial parasites of other bacteria; the relationship between structure and function of microorganisms and my involvement in advising, reviewing and regulating community water resources and associated programs in Northwest Ohio. My research has tried to blend bacterial physiology and electron microscopy into solving a problem in applied ecology---how to utilize bacteria to control populations of bluegreen algae.

The rationale of this approach is summarized nicely by a quotation from C. B. Huffakers (1974) book entitled Biological Control (Plenum Publishing Company): "If we are to reverse the trend toward an ever intensified overloading of the environment with polluting and highly toxic pesticides, we must show that biological control, combined with restricted usage of selective chemicals . . . and other integrative measures can, in fact, solve many of our pest problems without resort to disturbing and polluting chemicals."

That both bdellovibrios and bluegreen algae are very common in natural water habitats has been well-documented. This, plus definite evidence that bdellovibrios directly attack Phormidium and are capable of shutting off photosynthesis and of lysing these species of bluegreen algae in an agitated liquid environment, suggests that these bacteria may play a role in the control of bluegreen algal populations in their aquatic ecosystems. The natural tendency of B. bacteriovorus to interact with other microorganisms makes the relationship indicated here a more likely phenomenon to find in nature.

It is also important to consider the nature of the host organisms employed in this study. The blue green algae occupy a unique phylogenic and evolutionary niche in nature by being taxonomically placed between the bacteria and the higher plant forms; they have a unique motility which is not understood; they have a distinct structural organization; they are photosynthetic; they are autotrophic; they are extremely common to all water resources; they have a close association with a bacterial flora; and, they are the causative agents of a variety of important problems. The above characteristics make these organisms fascinating to work with and also economically important organisms to understand.

It should be emphasized that this research deals with understanding the relationship between bacteria and algae to control populations of algae. The data presented here will help to provide insight into biological control mechanisms---not only as to how algal populations can be biologically controlled---but also how parasitic microorganisms are involved in natural control of aquatic organisms. The aim of this research effort is to design a management program for severe algal problems. If such a practical endpoint can eventually be obtained, it will provide us with a more aesthetic water and a water of better quality because if bluegreen overgrowth can be prevented, the dissolved oxygen content of the water will be higher and the anaerobiasis that is so detrimental to water quality can be prevented.

My data as described in this report and its predecessor (Completion Report 414X-Bacterial Control of Aquatic Algae-1973) indicate that the potential is present for such a biological control system for the bluegreen algae. The following report describes the research accomplishments of the project up to July 1, 1976.

PROJECT OBJECTIVES

The following were the immediate and long-range objectives of this research project:

1. To determine the maximum effectiveness of the anti-algal factor produced by Bdellovibrio bacteriovorus 15143 in both eliminating and controlling bluegreen algal populations.

2. To examine the influence of environmental conditions on the production of the antialgal factor and its interaction with bluegreen algal cells.

3. To develop either facultative or host independent algal parasites, thereby eliminating the present need for bacterial host intermediates.

4. To develop a more effective bacterial parasite of algal populations.

Long-Range

1. To develop an effective means of utilizing bacteria to eliminate and control bluegreen algal populations in natural water resources.

CORRELATION OF OBJECTIVES WITH RESEARCH EXPERIMENTATION

I would like to indicate the major experiments that are described in this report as they relate to the overall objectives of this research.

1. The objectives examining maximum effectiveness of the antialgal factor (toxin) was approached in the following experiments:
   a) use of oxygen analyzer for measuring photosynthetic and respiration rate-speed of control of toxin on bluegreen algae;
   b) effect of heat treatment and autoclaving on the toxin;
   c) measurement of effect of toxin on different bluegreen algae;
   d) measurement of chlorophyll a and dry weight relationships in the presence of the toxin;
   e) effect of pH on antialgal activity of toxin;
   f) ultrastructural characterization of antialgal toxin against Phormidium luridum and Synechococcus species.
2. The objective examining of the influence of environmental conditions on the production of the antialgal factor was approached in the following experiments:
   a) experiments with different protein concentrations in bdellovibrio growth medium;
   b) protease levels in bdellovibrio growth medium;
   c) production of inhibitor in solid media.
3. The objective to identify and isolate this antialgal factor was approached in the following experiments:
   a) ultramembrane molecular weight filtration of heat-treated and normal bdellovibrio produced toxin;
   b) gel electrophoresis of the bdellovibrio culture supernatants;
   c) column chromatogratography of the antialgal toxin;

d) comparison of bdellovibrio toxin with other known chemical metabolic electron transport inhibitors;

e) *in situ* cytochemical comparison of bdellovibrio toxin with other chemicals affecting electron reduction reactions in *P. luridum*;

f) measurement of the effect of lyophilization of the antialgal toxin;

g) measurement of the effects of ultracold storage in the antialgal toxin.

4. Development of a host-independent algal parastie:

a) isolation of a host-independent bdellovibrio;

b) effect of medium change on bdellovibrio growth;

c) comparative effects of host-independent bdellovibrio whole cells, supernatant or autoclaved supernatant on *P. luridum*;

d) ultrastructure characterization of the algal parasite.

5. Development of a more effective parasite:

a) isolation and testing of more than 100 bacterial strains from Maumee Bay, Toledo, Ohio and other area water resources;

b) testing of 13 inhibitory strains with respect to bdellovibrio inhibition.

BACKGROUND

The reality of utilizing biological agents to control aquatic weeds and algae is upon us as was indicated in a recent (January, 1975) conference organized by USEPA solely to discuss this subject. As was pointed out by Sailer (1975), in the case of weed control, 8 out of 41 projects evaluated were noted as completely successful, while 9 gave substantial control, and 14 only partial control. This type of success rate clearly indicates that this concept is worth considerably more attention in the future.

This kind of approach, of artificially introducing a natural prey for an undesired host, provided the stimulus for investigating bacterial parasitism of algal populations.

The microbial interactions between bacteria and algae that result in algal lysis include lysis of both unicillular and filamentous bluegreen algae by a Myxobacter species (Shilo, 1970), a Cytophaga species (Stewart and Brown, 1969), and the lysis of various higher algae by members of the genus Pseudomonas (Mitchell, 1972). Daft and Stewart (1971) have reported additionally on myxobacteria that lyse bluegreen algae. By plaque assay, they demonstrated that one bacterial cell can cause algal lysis. They demonstrated the necessity of intact cells for inhibition of the algae to occur as cell filtrates had no effect on the algal cells. These authors have also reported to have had some success in limited field trials using myxobacteria to control Microcystis aeruginosa colonies (Fogg et al., 1973). Lysis in all of these cases had been cuased by an exocellular enzyme that functions only upon solid media. Until lysis of Phormidium by bdellovibrios in liquid systems was reported (Burnham and Stetak, 1972), all algal lytic processes needed a solid substrate.

Vibrios and related bacteria have been shown to lyse species of algae: (a) A vibrio was reported to attach to several species of the green algal genus, Chlorella by attaching to the cells and then lysing them (Mamkaeva, 1966; Starr and Seidler, 1971). The mechanisms for this activity are not resolved. (b) Recently, a report described an antibiotic substance produced by the genus Cellvibrio, that is capable of lysing vegetative cells of the bluegreen algae, Anabaena inaequalis (Granhall and Berg, 1972). It is particularly interesting that a bacterial protease had no effect on the algicide activity nor did boiling for 15 minutes. While pepsin had no effect, papain was two-thirds inhibitory. Molecular weight determinations by filtration support the above data indicating that the weight was between 1,000 and 10,000. The activity of this substance is apparently growth-dependent as darkness inhibited its lytic effect. Another difference from the lysis caused by Myxobacter and Cytophaga is that it occurred in liquid systems and the authors suggested that the substance might play a role in the algal control in natural ecosystems (Granhall and Berg, 1972).

Berland, Bonin and Maestrini (1972) looked at the toxicity of about 50 strains of bacteria for a variety of marine algae. Pseudomonas aeruginosa was found to be particularly inhibitory to Tetraselmis striata, a member of the Prasinophyceae. These authors concluded that it was not possible to state that bacteria or their byproducts are important in determining algal-bacteria relationships in the oceans.

Safferman and Morris (1962) demonstrated that actinomycete filtrates had considerable inhibitory activity against several strains of bluegreen algae. This work resulted in the suggestion that these antibiotic substances could be used as algicides. Sladekova and Sladek (1968) supported this idea of using bacterial secretion of antibiotics in the environment to control algae.

Similarly, a Bacillus brevis strain was shown to produce an extracellular

product that caused lysis of both several bluegreen algal species and several bacterial species (Reim et al., 1974). This non-enzymatic substance was quite heat-stable, or low molecular weight and could possibly be identified as an antibiotic similar to Gramicidin S. Reim et al. indicate that the utility of an antialgal antibiotic control system may be questionable due to the inability to achieve sufficient concentrations of the inhibitor in the general environment.

Investigations of a unique bacterium Bdellovibrio bacteriovorus have demonstrated the effectiveness of this small microorganism in destroying populations of host bacteria (Shilo, 1969; Starr and Baigent, 1966; Burnham, Hashimoto and Conti, 1968; Starr and Seidler, 1971). The occurrence of this organism in the natural environment is widespread and well-documented (Shilo, 1969).

The name Bdellovibrio bacteriovorus, first proposed by Stolp and Starr (Burnham and Robinson, 1974; Stolp and Starr, 1963), adequately expresses the principal characteristics of the organisms: "Bdello" is derived from the Greek word meaning "leech"; "vibrio" denotes its shape, and "bacteriovorus" indicates that it literally eats bacteria. Scherff, DeVay and Carroll (1966) were the first to show that B. bacteriovorus attacks on gram-negative bacteria resulting in the parasite actually penetrating into the host bacterium rather than remaining on the outside. These results were supported by Starr and Baigent (1966). Lepine et al. (1967) confirmed this endoparasitism in studying a B. bacteriovorus attacking Salmonella typhi obtained from a polluted river.

Burnham, Hashimoto and Conti (1968), investigating the penetration mechanisms in detail, showed that both physical and enzymatic actions combine to cause the localized breakdown of the host cell wall.

The evidence to date then supports the idea that B. bacteriovorus is an

endoparasite that eventually penetrates the host cell and multiplies within its confines. The cycle is completed on release of the new progeny from the host. Further refinement of the life cycle has been reported (Burnham, Hashimoto and Conti, 1970).

Antagonistic relationships between bacteria were reviewed by Stolp and Starr (1965), particularly in regard to the production of antimicrobial substances like enzymes, antibiotics and direct microbial attacks upon another cell. The Bdellovibrio bacteriovorus system is a classical example of this latter category. The mechanisms and enzyme interactions that explain how this parasitism is successfully completed are being unraveled, but are still not totally understood (Starr and Seidler, 1971), but a partial enzymatic understanding has resulted by a recent isolation of muramidases and proteases from bdellovibrio populations (Fackrell, Campbell, Huang and Robinson, 1972). These authors point out that by itself the bdellovibrio peptidase does not lyse living cells; only heat-killed cells are susceptible to enzyme degradation. They further demonstrate that the site of activity was the mucopeptide layer of both host, Spirillum serpens, and the parasite itself (Fackrell et al., 1972).

Bacteriolysis is not solely a characteristic of the bdellovibrio as a few other groups of bacteria, notably the myxobacteria, the cytophaga, and the actinomycetes are capable of lysing many bacterial strains as well as many algal species mentioned earlier. In studying the Myxobacter strain AL-1, Ensign and Wolfe (1966) described an enzyme possessing both proteolytic and cell-wall lytic activity. These two functions were inseparable upon purification, making the enzyme distinct from that isolated and purified from bdellovibrios (Fackrell, Campbell, Huang and Robinson, 1972; Dr. J. Robinson, personal communication). Review of the actinomycete lysis of other microbes

indicates that the responsible enzymes are peptidases (Stolp and Starr, 1966; Ghuysen, 1968), lending more support to the idea that the bdellovibrio factor, or aggressin causing the breakdown of the host organism is protein in nature, and possibly a specific peptidase in activity.

RESULTS

My laboratory found that active bdellovibrio cultures as well as culture supernatants (rendered cell-free by centrifugation and filtration) were capable of breaking down the Oscillatoria. The disintegration caused by the enzymatic secretions of the bdellovibrios is extensive, resulting in the loss of all cytoplasmic cell contents and eventual dissolution of the cell wall. In order that heterotrophic media be eliminated as a cause of this algal lysis, Oscillatoria cells were introduced into sterile tubes containing YP (yeast extrace and peptone) medium or nutrient broth and observed for a period of days. In spite of the fact that these cultures became overgrown with contaminating bacteria, the condition of the algae remained normal in terms of motility and cellular organization.

The condition of the Oscillatoria was observed immediately after adding the bdellovibrios to determine if any immediate physiological damage not visible by microscopy was occurring. The results indicate that both respiration and photosynthesis were within normal values after 1.5 hours' exposure to the bdellovibrios. Because it is very difficult to be sure of respiration and photosynthesis data when contaminating bacteria are present, cultures of the bluegreen algae Phormidium were employed as hosts for this experimentation with the bdellovibrios. The cultures of Phormidium are bacteria-free and allowed accurate measurement of oxygen uptake and evolution.

Four-day-old cultures of P. luridum grown in a mineral salts base were routinely utilized as hosts for the B. bacteriovorus 15143. If equal amounts of P. luridum culture are added to a 24-hour B. bacteriovorus culture, structural alterations in the bluegreen algae could be observed continuously until the algae lysed after four hours of interaction. These structural alterations included formation of refractile granules, intracellular spaces,

intercellular spaces, cell swelling, breakdown of the trichomes, and finally, disintegration of the algal cell. During the four-day period, the optical density of the mixed cultures gradually decreased. In all cases, the controls showed increasing cell densities throughout the week-long experiment, while the tubes with viable bdellovibrios showed total inhibition of growth and lysis of the P. luridum. Similar results can be observed for the cell-free culture supernatant of B. bacteriovorus 15143 (see Burnham et al., 1976 and the project completion report of Bacterial Control of Aquatic Algae [A-025-OHIO] by Burnham, 1973).

The bdellovibrio-P. luridum interaction showed measurable chlorophyll in the system to decrease markedly and the amount of protease activity in the cultures to increase (Burnham and Stetak, 1972; Burnham, Stetak and Locher, 1976).

Because of these structural and pigment changes, the photosynthetic activity of the P. luridum was monitored. Over 90 percent of all O_2 production by P. luridum or M. aeruginosa was inhibited by the B. bacteriovorus whole cells or by a cell-free supernatant prepared from a 24-hour bdellovibrio culture (Fig 1).

The Figure 1A illustrates that along with losing photosynthetic activity, the pigment chlorophyll a is lost from the cell or oxidized to a colorless form. Corresponding to pigment loss is a drop in turbidity and an associated loss in dry weight in the cultural interaction. This data was obtained using BdS (non-heat-treated) so that the bdellovibrio fraction did not contribute to the particulate weight.

Electron microscopy showed the gradual degradation of the photosynthetic thylakoids with a splitting of the paired photosynthetic membranes and the intermembranous space being filled with a low-density homogenous substance.

This splitting was shown to continue until the membranes disrupted (Burnham and Stetak, 1972; Burnham, 1973; Burnham and Sun, 1977 [see Appendix]). The end result was a mass of membrane fragments mixed with ribosomes and other cellular debris.

When the 24-hour culture supernatant was autoclaved, the photosynthetic inhibitory activity was retained. The autoclaved material inhibited over 90 percent of P. luridum and Microcystis aeruginosa O_2 activity in 15 minutes (Fig 1). Interestingly, a cell respiration was not inhibited (Fig 2). The inhibitor was shown to have a pH optimum of 9.5 and did not have any activity below pH 7.0 or above 11.0 (Fig 3). Ultramembrane filtration has shown the inhibitor to be less than 10,000 MW (Table 1).

Further research has led to activity being found at lower molecular weights. Using the Amicon Ultrafiltration cell, I have consistently identified inhibitory activities at 5000 MW, 1000 MW and 500 MW. The activity of the photorespiration phenomenon---discussed later---is lost below 10,000 MW. These data may suggest that more than one inhibitor is present affecting the P. luridum. I have not yet determined if it is two inhibitors or one inhibitor with perhaps a co-factor. Our experiments with dialysis (using 14,000 MW, 7500 MW and 3500 MW pore sizes) have always resulted in the loss of this activity. I believe these data indicate that a co-factor such as a divalent ion or other small compound is coupled to the larger inhibitor (between 5000 MW and 10,000 MW).

Gel filtration through Sephadex G-200 was also employed to purify the inhibitor. A simple peak was obtained at the equivalent molecular weight of 10-30,000. When fractions were pooled and concentrated by nitrogen gas evaporation and then tested for photosynthetic inhibiting activity, only a 10 to 20 percent inhibition was observed. Similar loss of activity was

observed after vacuum dialysis as well.

Disc gel electrophoresis (Ames) was employed to aid in the identification of protein that might be responsible for the antialgal activity. Several approaches to the problem of identifying this toxin were utilized.

1. Gels of 4xYP media, E. coli culture supernatant and bdellovibrio culture supernatant were run.
2. Gels of bdellovibrio supernatant fractions prepared by dialysis and ultracentrifugation were run and correlated with the oxygen inhibiting activity of the same fraction.
3. Gels were prepared of Phormidium luridum fractions, attempting to identify the presence of autolytic enzymes triggered by the anti-algal toxin activity contained in the bdellovibrio supernatant.

The procedure used the standard Ames techniques utilizing the standard gel system of Ornstein and Davis stacking at pH 8.9 (anodic system). The cathodic system as recommended by Ames employed the pH 4.3 system of Reisfeld, Lewis and Williams. Staining was with coomassie blue dye and destaining was carried out in 7% acetic acid.

Bands were found on 4xYP, and the culture supernatants, but the density of many of the bands was very light and made interpretation of the results difficult. The problem was how to get sufficient toxin or supernatant material in the gel buffer volume to enable dense banding.

When molecular weight filtrates were compared to the original supernatant, few protein bands were discernable. This was probably due to the slow concentration of the proteins I am trying to identify.

The principal problem encountered with identification of the P. luridum autolytic enzyme was a masking of the protein stains by the phycocyanin photosynthetic accessory pigment contained within these cells. The very dense bands

were discernable in spite of this diffuse blue coloration to the discs; however, all lighter bands were questionable in terms of reproducability. Dialysis of pigment prior to the disc gel electrophoresis did not help, as too many of the protein bands were also lost. Hopefully, many of the above described problems will be overcome by lyophilizing the toxin and the P. luridum proteins, thereby making it easier to obtain higher protein concentrations.

As can be seen in the data presented in Table IV, lyophilization was found to successfully preserve the toxin activity, especially in the untreated supernatant fraction. When the fractions were filtered through ultramembrane filters (Amicon), the amount of photosynthetic inhibitor activity (I) started to decrease. The fact that lyophilized samples retain their toxic activity can now be used to aid in purifying the active factor.

Also with regard to storing the toxin activity I have found that placing the bdellovibrio supernatant at -90°C caused only a small loss (11%) in photosynthetic inhibiting activity of the 4xYP supernatant over a 14-month test period (see Table V).

Approximately 50% of the inhibitory activity is lost when the bdellovibrio culture supernatant is subjected to pronase or trypsin protease activity (Burnham, Sun and Stetak, 1974).

I have determined that protein concentration plays a major role in the production of the inhibitor. To determine this fact, protein concentration was measured in cell-free supernatant fludis of B. bacteriovorus 15143 grown in four separate media: washed E. coli in buffer; E. coli in yeast extract; E. coli in yeast extract and peptone; and E. coli in 4x yeast extract and peptone, which I found to be 0, 45, 60 and 190 ug/ml, respectively.

The sharing of electron transport, components and pathways between photosynthesis and respiration has been postulated (2, 28). The data presented

for P. luridum would support this concept.

One of the major problems that our present methodology does not permit is a direct cytochemical measurement of photosynthetic transport with the total blockage of respiration. As described in our papers (Sun et al., 1975; Sun and Burnham, 1977 [see Appendix]), copper ferricyanide does the reverse in the presence of light, demonstrating respiratory electron transport with the blockage of photosynthesis. Until a unique cytochemical agent is designed, the definite cytochemistry of photosynthesis will be impossible.

Most of the effects of the chemical inhibitors used in this investigation are known (see Materials and Methods). However, the inhibitory effects of BdAS which so profoundly affects physiological activities of cyanobacteria is still an unknown process. Autoclaved bdellovibrio toxin caused the P. luridum, under lighted conditions, to undergo photorespiration, instead of photosynthesis (8). In comparing the results of BdAS treatment with those of DCMU, KCN, ouabain, rotenone and polymyxin B, the bdellovibrio toxin has to be regarded as unique and very potent.

Fig 4 shows that photosynthetic inhibition by both the normal and heat-treated supernatant fluids directly depended upon protein concentrations. We previously felt that inhibition was related to protease activity of the bdellovibrio (Burnham, Stetak and Locher, 1976), and therefore, compared protease activity in these respective supernatant fluids. The results are shown in Fig 5. This figure indicates that there is no relationship of protease activity to the photosynthetic inhibitory activity shown in Fig 4. This data emphasizes why the requirement for protein should be investigated in the future if this inhibitor is to be successfully utilized against algae.

Measurement of the photosynthetic inhibition of B. bacteriovorus 15143 by the Clark electrode system produced an unexpected finding as to the effects of

the bdellovibrio cell-free supernatant fluid in the P. luridum. When protein concentration was increased, the amount of inhibition of oxygen production after 1 h. exposure increased beyond the 100% level (Fig 2). In other words, not only was the photosynthesis shut down by the inhibitor, but the cell respiratory rate was increased at the same time. This phenomenon was light-dependent as is indicated in the dark portion of the curve showing the respiratory rate to return to a more normal rate. Autoclaved or normal supernatant behaved similarly. Host-independent supernatant produced a similar result (see Fig 6). It should be emphasized that control P. luridum were suspended in 4xYP for 1 h p prior to being monitored for O_2 activity and did not show this phenomenon.

In eukaryotic chloroplasts, photorespiration can be explained metabolically by the biosynthesis and metabolism of glycolate. The total sequence has been referred to as the glycolate pathway. Quantitatively, glycolate metabolism and photorespiration seem similar in all photosynthetic algae and cyanobacteria.

Biochemical details of glycolate biosynthesis have remained obscure, but current research favors the hypothesis for its formation from ribulose diphosuphoric acid (RuDP). The action of RuDP oxygenase and RuDP carboxylase results in a competition between O_2 and CO_2 for the common substrate RuDP. Under the light, activity of RuDP oxygenase results in O_2 consumption. The role of light in the reaction is indirect through the photosynthetic generation of RuDP. There is a possibility that BdAS either denatures RuDP carboxylase or unmasks the reactive sites of RuDP oxygenase resulting in a strong photorespiration activity completely obscuring the photosynthetic activity of P. luridum in light.

It was proposed that glycolate may be formed by a peroxidation of a thiamine pyrophosphate-glycolaldehyde (TPP-C_2) complex. It has been demonstrated that any strong oxidant such as ferricyanide or H_2O_2 will oxidize TPP-C_2 to

glycolate *in vitro*. This may explain why the degree of ferricyanide reduction in BdAS-treated cells is even higher than in the controls.

Autoclaved bdellovibrio toxin is considered a possible means for cyanobacterial control. Without a doubt, environmental control through biological means would be a most desirable alternative to the unfavorable side effects of chemical inhibitors.

This result is one of the more exciting findings in this project and indicates the potential for complete algal shutdown by these bdellovibrio substances if conditions are correct. Obviously, the next phase of this project must be isolation of the factor and defining its inhibitory properties under conditions expected in the natural environment with an examination of the relationship between photorespiration and algal death.

Dichloromethylurea, potassium cyanide and photosynthetic inhibitor produced by *Bdellovibrio bacteriovorus* are compared for their effects on *Phormidium luridum* with regard to morphology, ultrastructure, photosynthetic O_2 evolution and *in vivo* ferricyanide reduction. The effects of DCMU and the bdellovibrio factor closely resemble each other, suggesting that the bdellovibrio effect may be localized at photosystem II (see Appendix). *Phormidium luridum* incubated with DCMU or bdellovibrio factor for 1 hr showed 100% inhibition of O_2 evolution. Light-dependent ferricyanide reduction was also inhibited. Both agents had no effect on dark respiratory O_2 uptake or dark ferricyanide reduction. Alteration of the internal fine structure of *Phormidium luridum* was observed through the electron microscope after exposure of cells to DCMU or bdellovibrio factor for one to two days. In both cases, separation of adjacent photosynthetic lamellae occurred, spaces or intrathylakoidal vacuoles which, devoid of cytoplasmic material, can be observed between the splitting lamellae.

The number, as well as the area of these empty spaces progressively increased with time. On the fifth or sixth day, approximately 50% of the entire internal volume of cells were occupied with empty spaces. Also, some photosynthetic membranes were rounded up into spherical shape with part of the cytoplasmic materials being enclosed within these spherical structures (Sun, Locher and Burnham, 1975). The identical change in cell ultrastructure triggered by different chemical compounds is believed due to autolytic activity of bluegreen algal cells in response to the stimulus provided by DCMU or the bdellovibrio factor. Further, although the pattern of ultrastructural breakdown was similar to these two agents within species, it differed between species. For example, M. aeruginosa responded to these agents with disarrangement and disintegration of photosynthetic membranes (Sun, Locher and Burnham, 1975).

Because B. bacteriovorus 15143 had no available host-independent (HI) strain, it was necessary to isolate our own mutant strain (Note: The American Type Culture Collection had previously collected such a strain but when inquiry was made, their strain had become contaminated and had died out). This was accomplished by serial transfer in YP medium of the host-dependent strain previously grown on E. coli 15144 in YP medium for several months on a daily basis. By not adding fresh E. coli to this medium a constant pressure to saprophytic growth was maintained with the eventual emergence of a successful host-independent strain.

After isolation of this host-independent mutant in late 1974, a growth curve was prepared representing its development in these media: 1xYP, 4xYP and 4xYP supplemented with .002 M $CaCl_2$ and $MgCl_2$. This latter media was utilized because of a noted tendency for some of the organisms to spheroplast during the repreoductive phase of growth. Fig 7 shows the comparative curves

illustrating that although somewhat higher density growth was reached by the bdellovibrios with added ions, the rate of growth during lag phase was not affected.

Inhibition of P. luridum growth was tried with the bdellovibrio with results shown in Fig 8. This curve illustrates (a) that only the viable independent cells were effective in limiting algal growth---a result in contrast to the host-dependent strain where supernatant factors were only partially inhibitory to the algal growth.

The results were confirmed by O_2 analysis which showed that supernatant fractions possessed little inhibitory activity. Only recently did it occur to me that the pH of the supernatant mixture with the P. luridum might be below the limit for inhibiting activity. When pH was raised to 8.8, O_2 inhibition was total with the added effect of photorespiration---a better than expected result.

Host-independent bdellovibrios have been reported before (Shilo and Bruff, 1965) to resemble morphologically their parent strains and possess an active ability to produce a potent exocellular protease. The B. bacteriovorus 15143 HI strain isolated also possessed mobility, a vibrio shape and an active proteolytic enzyme. Electron microscopic examination of the HI bdellovibrio revealed that it contains many large dense particles not seen in the parent cell which I believe at present to be polyphosphate structures (Fig 9). No difference is observed in the cell envelope of the mutant, and the polar flagellum is ensheathed with lipopolysaccharide like its parent.

Huang and Starr, 1973, have determined that one independent strain of bdellovibrio produces exocellular protease, peptidase, lysozyme-like enzyme and lipase, but no mention was made of the enzymes' ability to withstand heat treatment. Their use of autoclaved host cells insured that host cell

breakdown was not autolytic.

Similarly, Enjelking and Seidler, 1974, identified such enzymes and added nucleases to the list of bacteriolytic agents produced by the host-independent bdellovibrio.

SOLID MEDIA TECHNIQUES AND OTHER INHIBITORY BACTERIA

Almost everyone who has worked with bluegreen algae knows the teanciousness with which bacteria are associated with these organisms. It is extremely difficult to separate the bluegreen algae completely from its symbiotic flora. This bacteria/bluegreen algae relationship has been studied (Ward and Moyer, 1967; Lange, 1967; Lange, 1071; Nalewajko and Lean, 1973; Fitzgerald, 1969; Belly, Tansey and Brock, 1973) with documentation of the secretory relationship of the algae to the CO_2 producing role of the associated bacterial flora. Most of the reports describing this relationship are either taxonomic or indicate a positive role (symbiosis) for each member of the association. Lange (1967) reported that upon maximal growth of the associated bacteria, the bacteria successfully competed for metal chelates (ferric citrate, etc.), resulting in the bleaching and deterioration of the culture. Fitzgerald (1969) described an antagonistic relationship between M. aeruginosa and unidentified bacteria that prevented the growth of the bluegreen algae. Unfortunately, isolation and characterization of these algal inhibitory agents was not possible.

Figures 10 and 11 show that the addition of E. coli to P. luridum agar plate cultures did appreciably stimulate the development of the bluegreen cells. Phase contrast microscopic observations indicate that the P. luridum culture caught up after a week. Some enhancement of the bacterial number per square centimeter was noted after 24 hr, but this appeared to decline slowly. The experiment indicated that artificial bacterial/algal relationships could

be studied and might serve as models for the development of successful bacterial antagonists.

As part of a project for the Toledo-Lucas County Port Authority (Fraleigh, Burnham, Gronau, Kovacik, Tramer, 1975) studying the water quality of Maumee Bay and Lake Erie, I was able to isolate approximately 150 bacterial strains. Approximately 60 were tested for antialgal activity, and from this number, 12 algal inhibiting strains were selected and kept. The fact that thes these bacteria were present in natural water environment and were capable of inhibiting algae in both a plate (Fig 15) or a liquid environment makes flora substitution of algal antagonists an exciting route to investigate as a potential control mechanism.

For preparation of the plate lysis technique, 14 liter batches of P. luridum or Anabaena cylindrica were centrifuged to a slurry and washed once in phosphate buffer and resuspended in agar. This heavy green mixture (3 ml) was then pour-plated over a 15 ml plain agar base. Samples were then streaked on this medium and clearing zones examined for inhibitory bacteria. This technique has been surprisingly effective in producing colonies of bacteria capable of degrading and surviving on the bluegreen algal cells with no protein present.

Table 3 lists some of the characteristics of these isolates along with possible isolate identification. When these strains were tested for ability to inhibit photosynthesis and growth, the results showed potential for use as inhibitors. The gram-positive inhibiting bacteria were eliminated subsequently as they were invariably spore formers liberating bacitracin or related antibiotic during the sporulating process. They, therefore, would not be acceptable parasites as spores are essentially inert until they undergo eventual germination.

The G-2 isolate has been extensively tested and a curve (Figure 12) shows that inhibiting photosynthetic activity did occur as did inhibition of growth. I am planning to use this organism in media substitution experiments using carbohydrates to replace the protein, hoping to induce inhibition in non-protein environments.

PRESENT STATUS OF ALGAL CONTROL BY INHIBITORY BACTERIA

This project has uncovered much information concerning the nature of the bdellovibrio produced inhibitors of bluegreen algae. Much of this indicates that the inhibition is unique and effective. However, I believe there are significant problems which have also been raised by the research which remain to be answered. Probably the major problem in terms of beginning to design the system to be more effective in less controlled environments is that of protein requirement for inhibitor production. It is impossible that such a system could be effective in the natural environment. In the Phase II segment of this research, I have approached this question directly and hopefully can evolve a carbohydrate dependency instead.

Survival of the bdellovibrio parasite is a second major problem. The 15143 B. bacteriovorus strain presently used in the majority of experiments does not survive well over long periods in fresh water. I hope that the host independent form can be improved in this regard by selective pressures. If it can not, I am intending to utilize some of the other bacteria isolated as part of this study to be the center of effort. This direction makes sense as these bacteria have a natural habitat of fresh water and in the case of the G-2 organism, have an excellent potential for non-protein dependent algal control.

In summary, there has been considerable data generated through this project which contributes to the understanding of bacterial bluegreen algal

interactions. This developing data is necessary to progress toward our goal of an effective algal controlling microorganism.

REFERENCES

1. Belly, R. T., Mr. R. Tansey and T. D. Brock. Algal excretion of ^{14}C-labeled compounds and microbial interactions in Cyanidium caldarium mats. J. Plycol. 9:123-127.

2. Berland, B. R., D. J. Bonin and S. Y. Maestrini, 1972. Are some bacteria toxic for marine algae? Marine Biol. 12:189-193.

3. Burnham, J. C. 1973. Bacterial control of aquatic algae. Project Completion Report No. 414X. State of Ohio Water Resources Center, U. S. Department of Interior.

4. Burnham, J. C., Hashimoto, T. and Conti, S. F. 1968. Electron microscopic observations on the penetration of Bdellovibrio bacteriovorus into gram-negative bacterial hosts. J. Bacteriol. 96:1366-1381.

5. Burnham, J. C. and J. Robinson. 1973. The genus and species of Bdellovibrio. In N. Gibbons and R. E. Buchanan, eds. Bergey's Manual of Determinative Bacteriology, 9th Edition. In press.

6. Burnham, J. C. and T. Stetak. 1972. Observations on the interaction of Bdellovibrio bacteriovorus with two species of blue-green algae. Abs. Ann. Mtg. Am. Soc. Microbiol. p. 33 (G18).

7. Burnham, J. C., Stetak, T., and Locher, G. 1976. Extracellular lysis of the blue-green algae Phormidium luridum var. olivacea by Bdellovibrio bacteriovorus. J. Phycol. 12:306-313.

8. Burnham, J. C., D. C. Sun and T. Stetak. o974. Biological properties of a Bdellovibrio-produced inhibitor of blue-green algae. Abs. Ann. Mtg. Am. Soc. Microbiol. G255.

9. Daft, J. J. and Stewart, W. O. P. 1971. Bacterial pathogens of fresh water blue-green algae. New Phytol. 70:819-829.

10. Engelking, H. M. and R. J. Seidler. 1974. The involvement of extracellular enzymes on the metabolism of Bdellovibrio. Arch. Microbiol. 95:293-304.

11. Ensign, J. C. and R. S. Wolfe. 1966. Characterization of a small proteolytic enzyme which lyses bacterial cell walls. J. Bacteriol. 91:524-534.

12. Fackrell, H. B., G. K. Campbell, J. C. C. Huang and J. Rob inson. 1972. Evidence for a lytic enzyme produced by Bdellovibrio bacteriovorus. 6-5-S. Can. J. Microbiol. 18:281-287.

13. Fitzgerald, G. P. 1969. Some factors on the competition or antagonism among bacteria, algae, and aquatic weeds. J. Phycol. 5:351-359.

14. Fogg, G. E, W. O. P. Stewart, P. Fry and A. E. Walsby. 1973. The Blue-Green Algae, Academic Press, N. Y. pp. 297.

15. Fraleigh, P. C., J. C. Burnham, G. H. Gronau, T. L. Kovacik, and E. J. Tramer. 1975. Maumee Bay Environmental Quality Study. Toledo-Lucas County Port Authority; Toledo, Ohio pp. 288.

16. Ghuysen, J. M. 1968. Use of bacteriolytic enzymes in determination of wall structure and their role in cell metabolism. Bact. Rev. 32: 425-464.

17. Granhall, U. and B. Berg. 1972. Antimicrobial effects of Cellvibrio on blue-green algae. Arch. Microbiol. 84:234-242.

18. Huang, J. C. C. and M. P. Starr. 1973. Possible enzymatic bases of bacteriolysis by Bdellovibrios. Arch. Microbiol. 89:147-167.

19. Lange, W. 1967. Effect of carbohydrates on the symbiotic growth of planktonic blue-green algae with bacteria. Nature 215:1277-1278.

20. Lange, W. 1971. Enhancement of algal growth in cyanophyta -bacteria systems by carbonaceous compounds. Can. J. Microbiol. 17:303-314.

21. Lepine, P. M., A. Guelin, J. Sisman, and D. Lamblin. 1967. Etude au microscope electronique de la lyse de Salmonella par Bdellovibrio bacteriovorus.

22. Mamkaeva, K. A. 1966. Studies of lysis in cultures of Chlorella. Microbiology (USSR: English Transl.) 35:724-728.

23. Mitchell, R. 1972. Water Pollution Microbiology. Wiley Interscience, New York.

24. Nalewajko, C., and D. R. S. Lean. 1972. Growth and excretion in planktonic algae and bacteria. J. Phycol. 8:361-366.

25. Reim, R. L., Shane, M. S. and Canon, R. E. 1974. The characterization of a bacillus capable of blue-green bactericidal activity. Can. J. Microbiol. 20:981-986.

26. Safferman, R. A. and M. E. Morris. 1962. Evaluation of natural products for algicidal properties. Appl. Microbiol. 10:289-292.

27. Safferman, R. S. and M. E. Morris. 1964. Control of algae with virus. J. Am. Water Works Assoc. 56:1217-1224.

28. Sailer, R. I. 1975. Principles and techniques of biolgoical control. In Proceedings of a Symposium on biological Control for Water Quality Enhancement. Gainesville, Florida, January 1975.

29. Scherff, R. H., J. E. DeVay and T. W. Carroll. 1966. Ultrastructure of host-parasite relationships involving reproduction of Bdellovibrio bacteriovorus in host bacteria. Phytopathology. 56:627-632.

30. Shilo, M. 1969. Morphological and physiological aspects of the interaction of Bdellovibrio with host bacteria. Curr. Top. Microbiol. Immunol. 50:174-204.

31. Shilo, M. 1970. Lysis of blue-green algae by Myxobacter. J. Bacteriol. 104:453-461.

32. Shilo, M. and B. Bruff. 1965. Lysis of gram-negative bacteria by host-independent ectoparasitic Bdellovibrio bacteriovorus isolates. J. Gen. Microbiol. 40:317-328.

33. Sladeckova, A. and V. Sladeck. 1968. Algicides---friends or foes? In Algae, Man and the Environment. pp. 441-458. Syracuse University Press, New York.

34. Starr, M.P. and N. L. Baigent. 1966. Parasitic interaction of Bdellovibrio bacteriovorus with other bacteria. J. Bacteriol. 91:2006-2017.

35. Starr, M. P. and R. J. Seidler. 1971. The Bdellovibrios, p. 649-678. In C. E. Clifton (ed.) Ann. Rev. Microbiol. Vol. 25. Annual Reviews, Inc., Palo Alto, California.

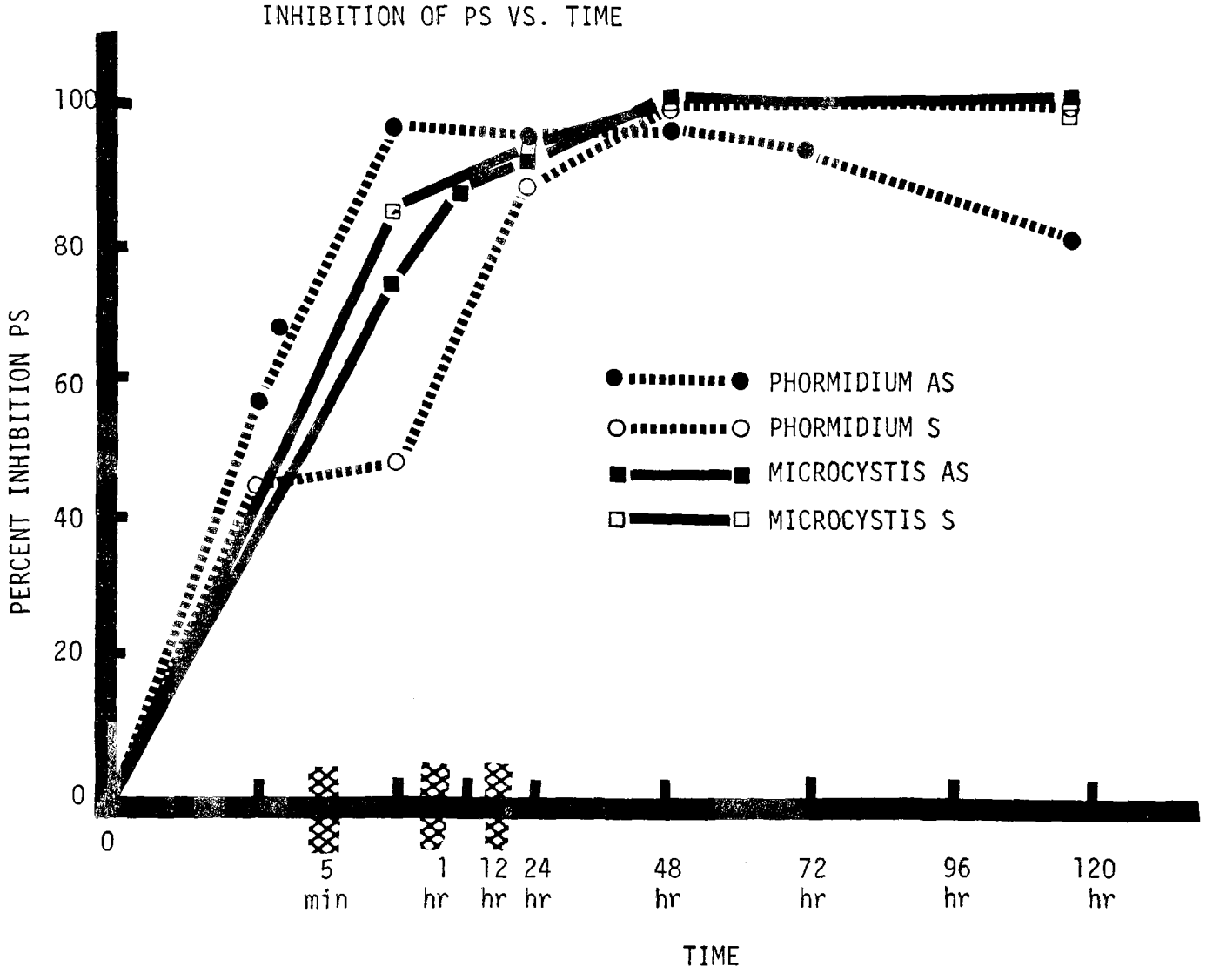
36. Stewart, J. R. and R. M. Brown. 1969. Cytophaga that kills and lyses algae. Science 164:1512-1524.

37. Stolp, H. and M. P. Starr. 1963. Bdellovibrio bacteriovorus gen. et sp. n., a predatory, ectoparasitic, and bacteriolytic microorganism. Ant. van Leeuwenhoek. J. Microbiol. Serol. 29:217-248.

38. Sun, D. C., and J. C. Burnham. 1975. Comparison of photosynthetic inhibitors in spheroplasts and whole cells of Phormidium luridum. Agst. Ann. Mtg. Soc. Microbiol. 171.

39. Ward, C. H. and J. E. Moyer. 1966. Ecological relationships between bacteria and algae in mass culture. Paper presented at AIBS and NASA sponsored conferences on biogenetic systems, Washington D. C. Nov. 15-16, 1966.

Figure 1 A curve showing the effect of B. bacteriovorus cell-free supernatant(s) and autoclaved cell-free supernatant (AS) on the photosynthetic (oxygen evolution) ability of P. luridum and M. aeruginosa. The O_2 percent inhibition level was represented by the mean photosynthesis of control cultures.


INHIBITION OF PS VS. TIME
PERCENT INHIBITION PS
100
80
60
40
20
0
0
5 min
1 hr
12 hr
24 hr
48 hr
72 hr
96 hr
120 hr
TIME
PHORMIDIUM AS
PHORMIDIUM S
MICROCYSTIS AS
MICROCYSTIS S

1

Figure 1A This graph shows that the bdellovibrio supernatant causes a loss in both chlorophyll a and particulate dry weight from an initially healthy 96 hr culture of P. luridum. This activity can be measured with either BdS or autoclaved BdS (BdAS), indicating that the majority of the lytic activity is autolytic in nature. Structural results support this observation (see Appendix).

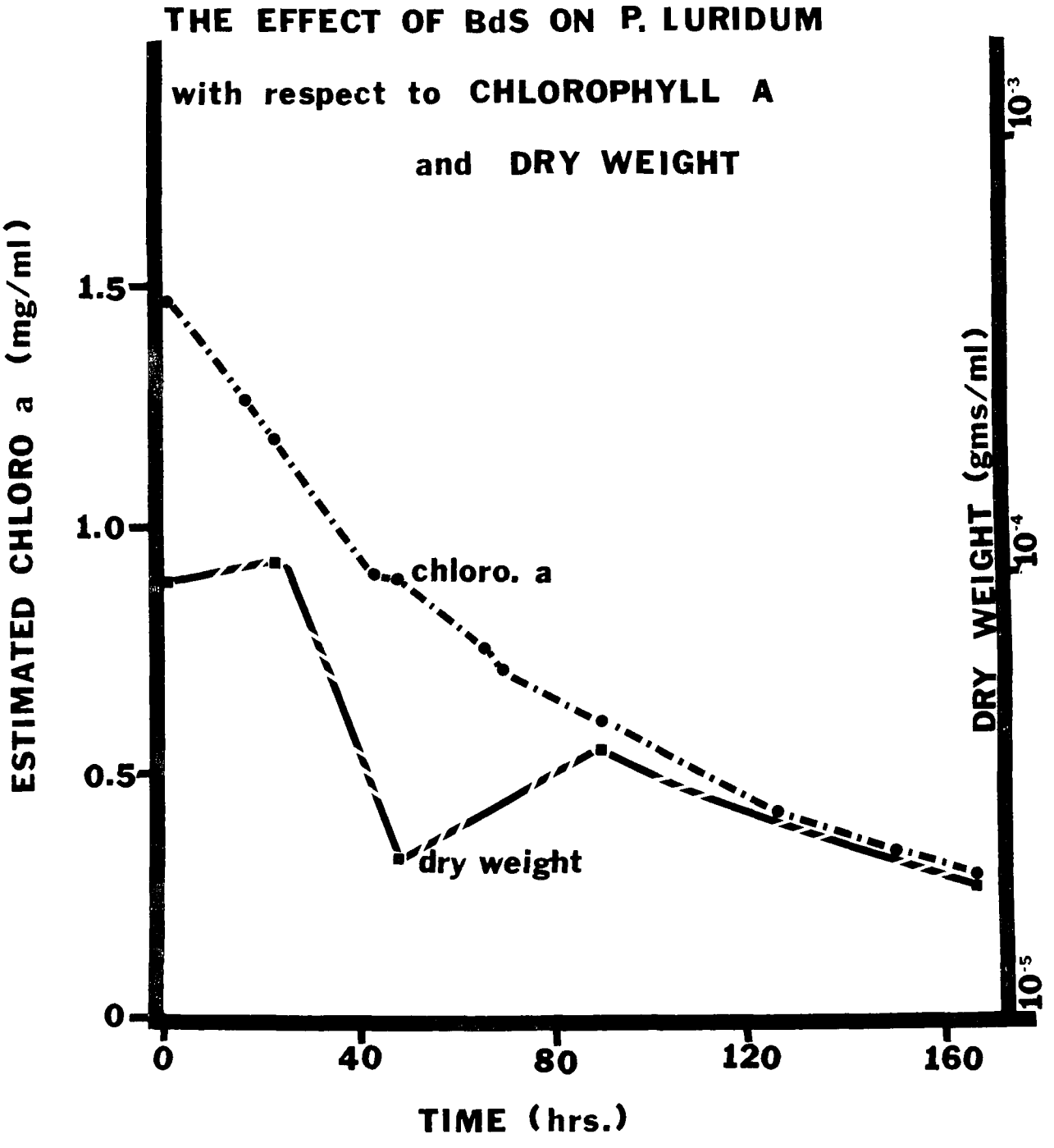
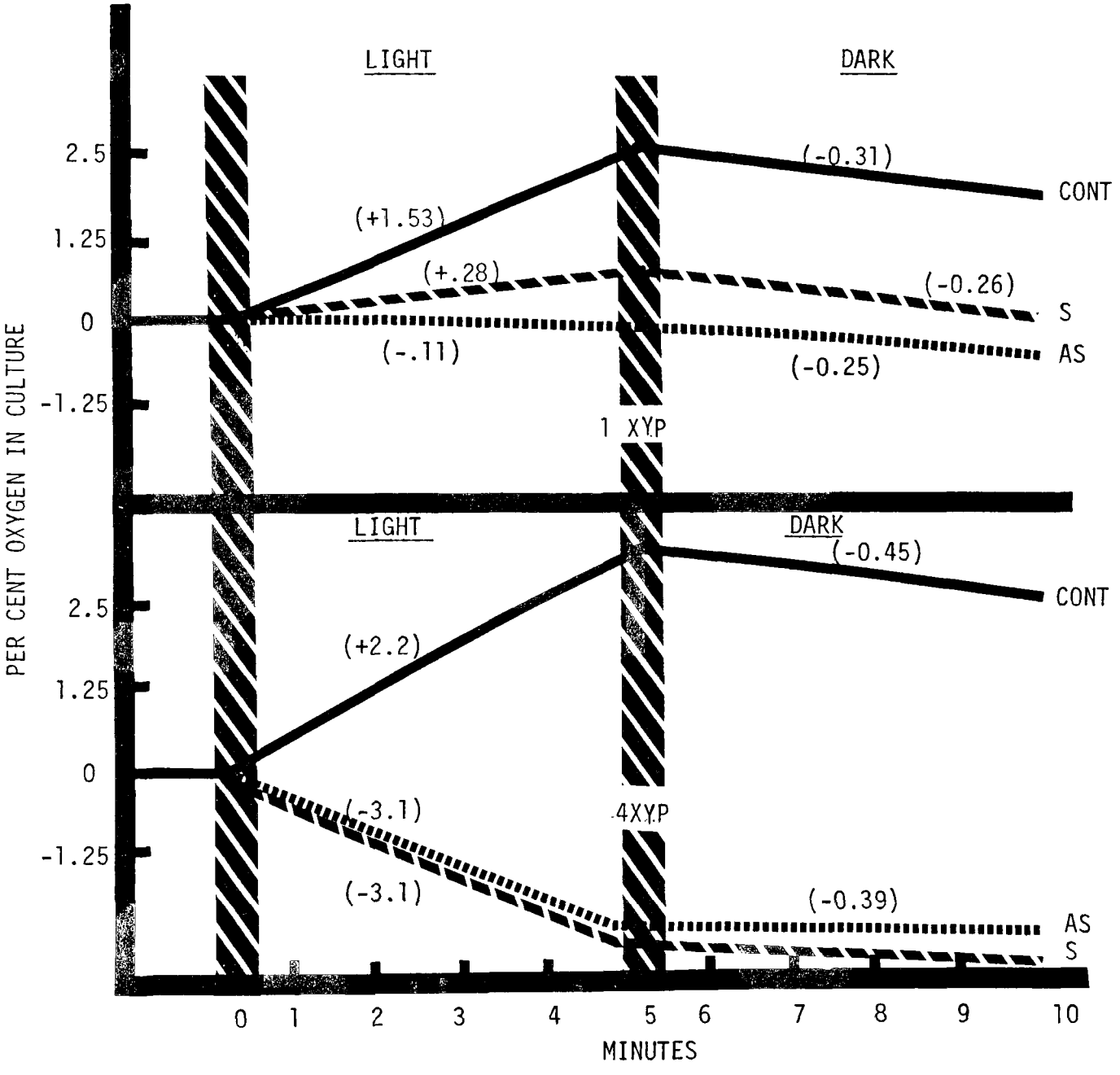

THE EFFECT OF BdS ON P. LURIDUM
with respect to CHLOROPHYLL A
and DRY WEIGHT
ESTIMATED CHLORO a (mg/ml)
DRY WEIGHT (gms/ml)
1.5
1.0
0.5
0
10^{-3}
10^{-4}
10^{-5}
chloro. a
dry weight
0
40
80
120
160
TIME (hrs.)

Figure 2 A curve illustrating the effect of B. bacteriovorus on P. luridum photosynthesis and respiration. The upper curve was representative of the inhibition of B. bacteriovorus when grown in 1xYP medium (moderate protein concentration), while the lower curve was representative of inhibition when they were grown in 4xYP medium (high protein concentration). The light and dark 5-minute cycles are indicated to separate photosynthesis from respiration. The numbers indicate the $ul O_2$/ug Chla/hr given off or taken up by the P. luridum cell. Note the photorespiration stimulated by the 4xYP fraction in the light.

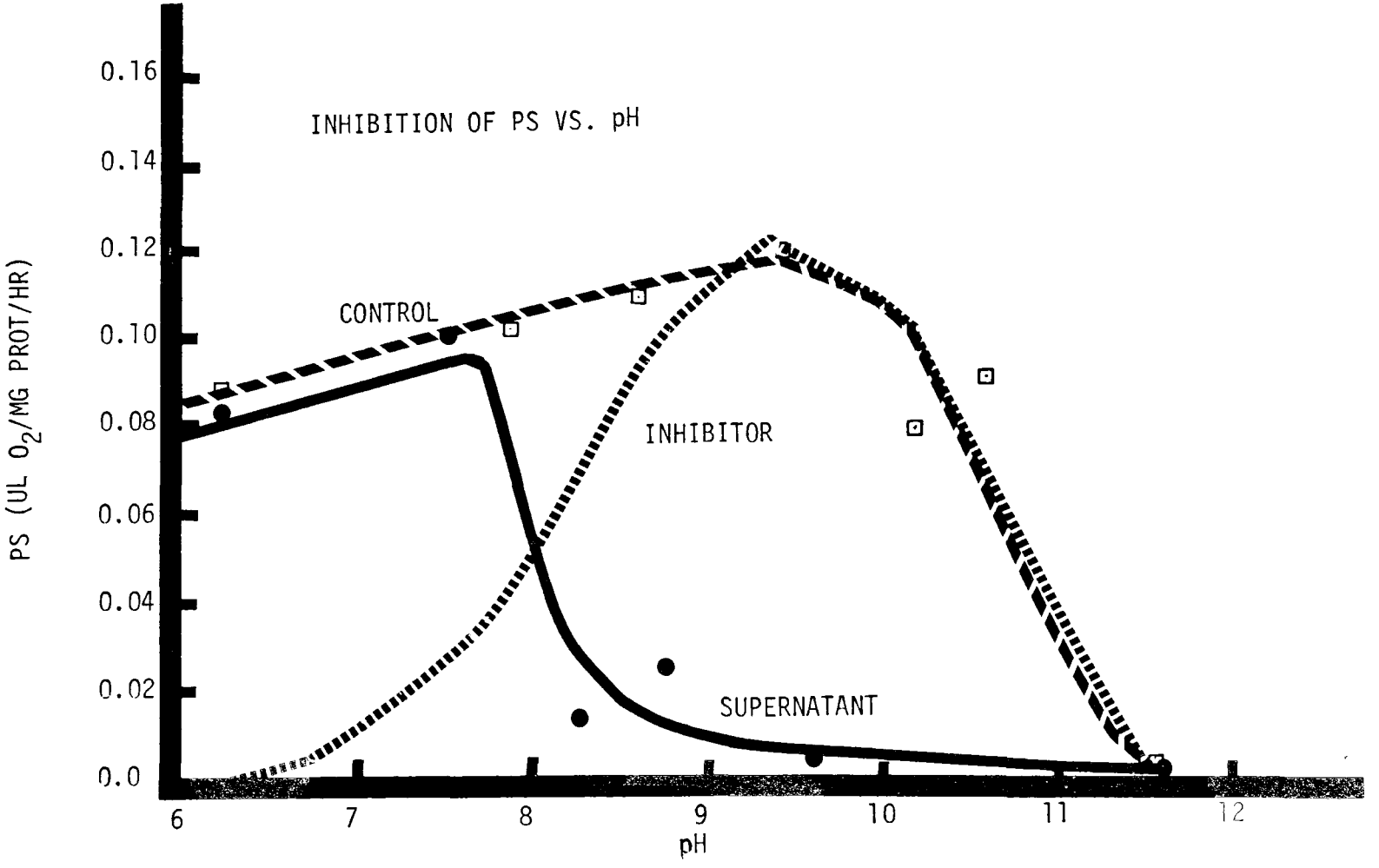
INHIBITION OF P. LURIDUM PHOTOSYNTHESIS* AND RESPIRATION*

* (#) = $ul O_2$/ug Chla/hr



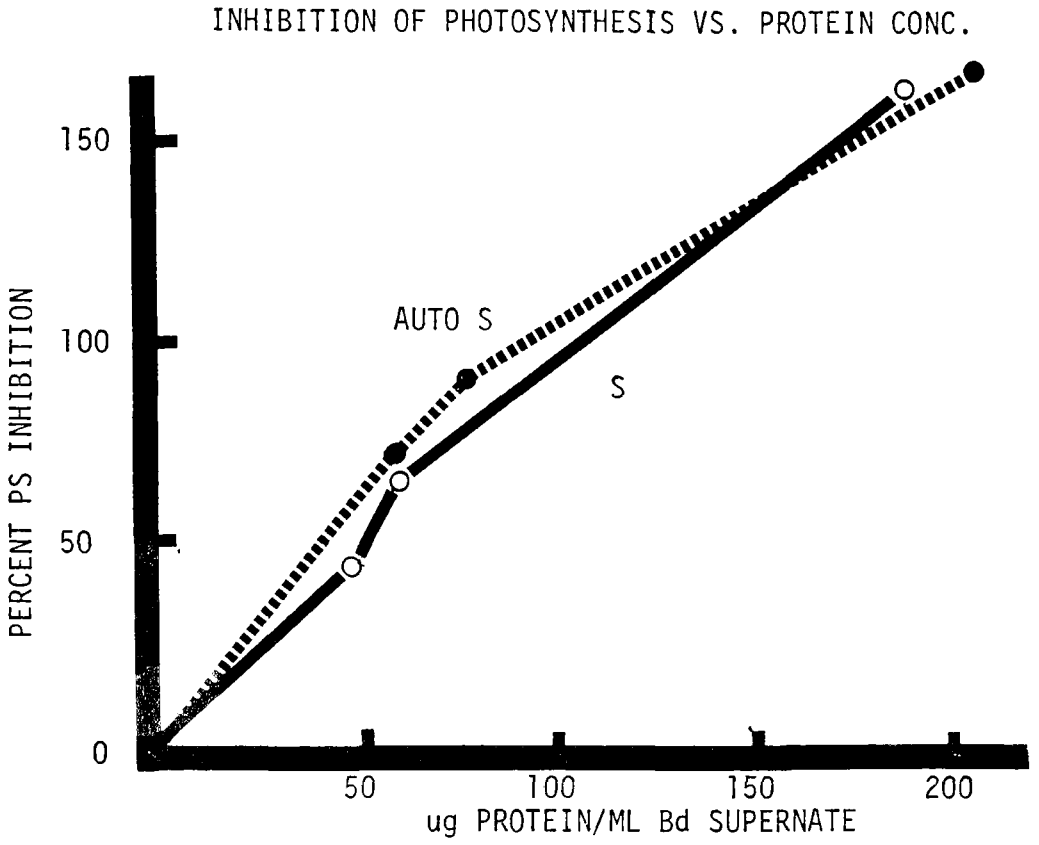
2

Figure 3 A curve illustrating the effect of pH on the photosynthetic inhibitory activity of a Bdellovibrio bacteriovorus cell-free supernatant. Maximum inhibitory activity was seen from pH 9 to pH 10. When the difference between control P. luridum photosynthesis and that of P. luridum exposed to inhibitor for 1 hr was plotted, a peak of inhibitory activity was obtained.


INHIBITION OF PS VS. pH
PS (UL O_2/MG PROT/HR)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.0
CONTROL
INHIBITOR
SUPERNATANT
6
7
8
9
10
11
12
pH

3

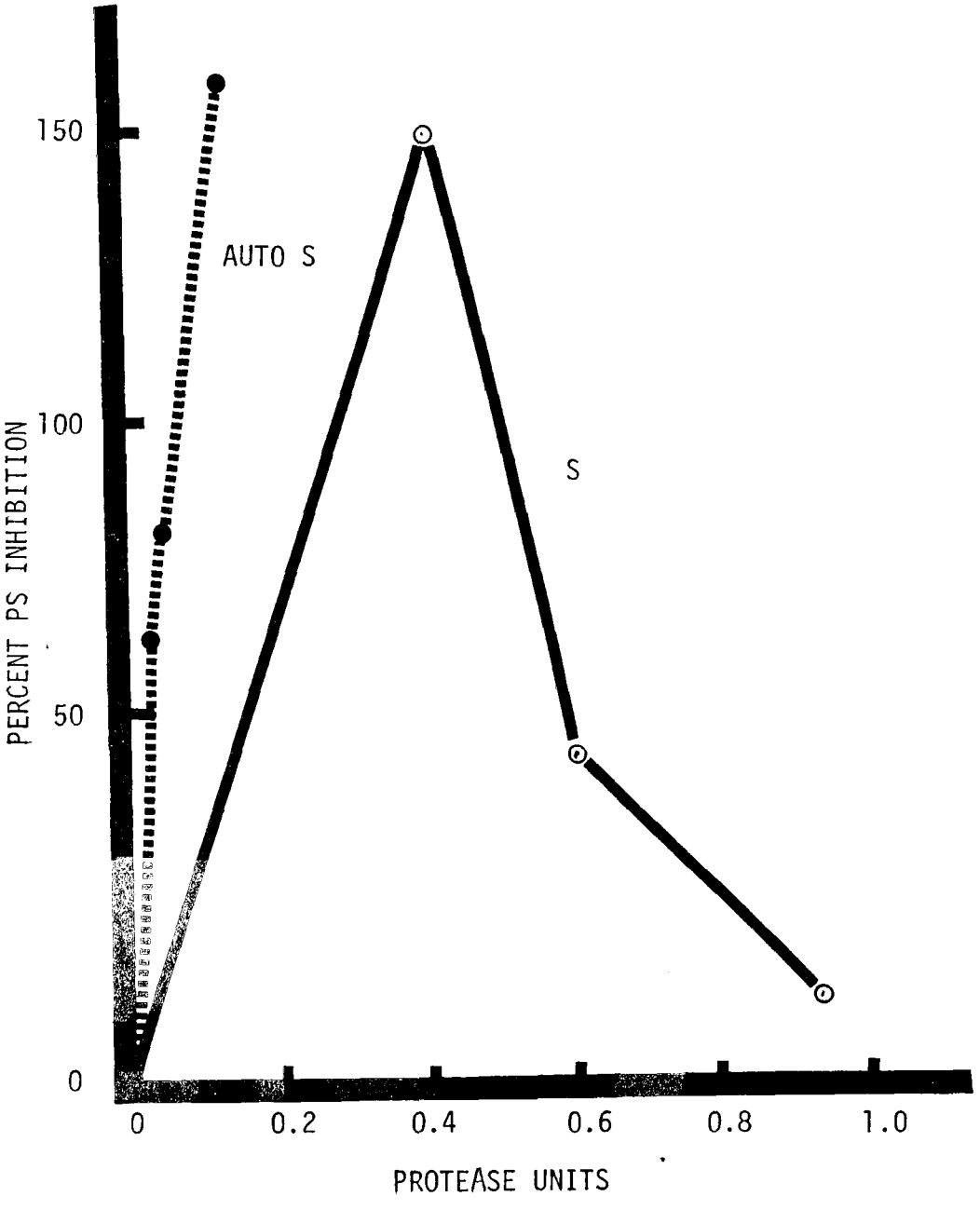
Figure 4 A curve showing the effect of protein concentration in the B. bacteriovorus growth medium on the percent inhibition of photosynthesis of P. luridum after 1 hr exposure. S = cell-free bdellovibrio culture supernatant; AS=autoclaved cell-free bdellovibrio culture supernatant. Percent inhibition greater than 100 indicates stimulation of photorespiration as indicated in Figure 7.


INHIBITION OF PHOTOSYNTHESIS VS. PROTEIN CONC.
PERCENT PS INHIBITION
150
100
50
0
AUTO S
S
50
100
150
200
ug PROTEIN/ML Bd SUPERNATE

4

Figure 5 A curve showing that little relationship exists between the amount of protease in cell-free bdellovibrio supernatant (S) as autoclaved supernatant (AS) and the amount of inhibition of P. luridum photosynthesis. Note that autoclaving apparently destroyed much of the protease activity.

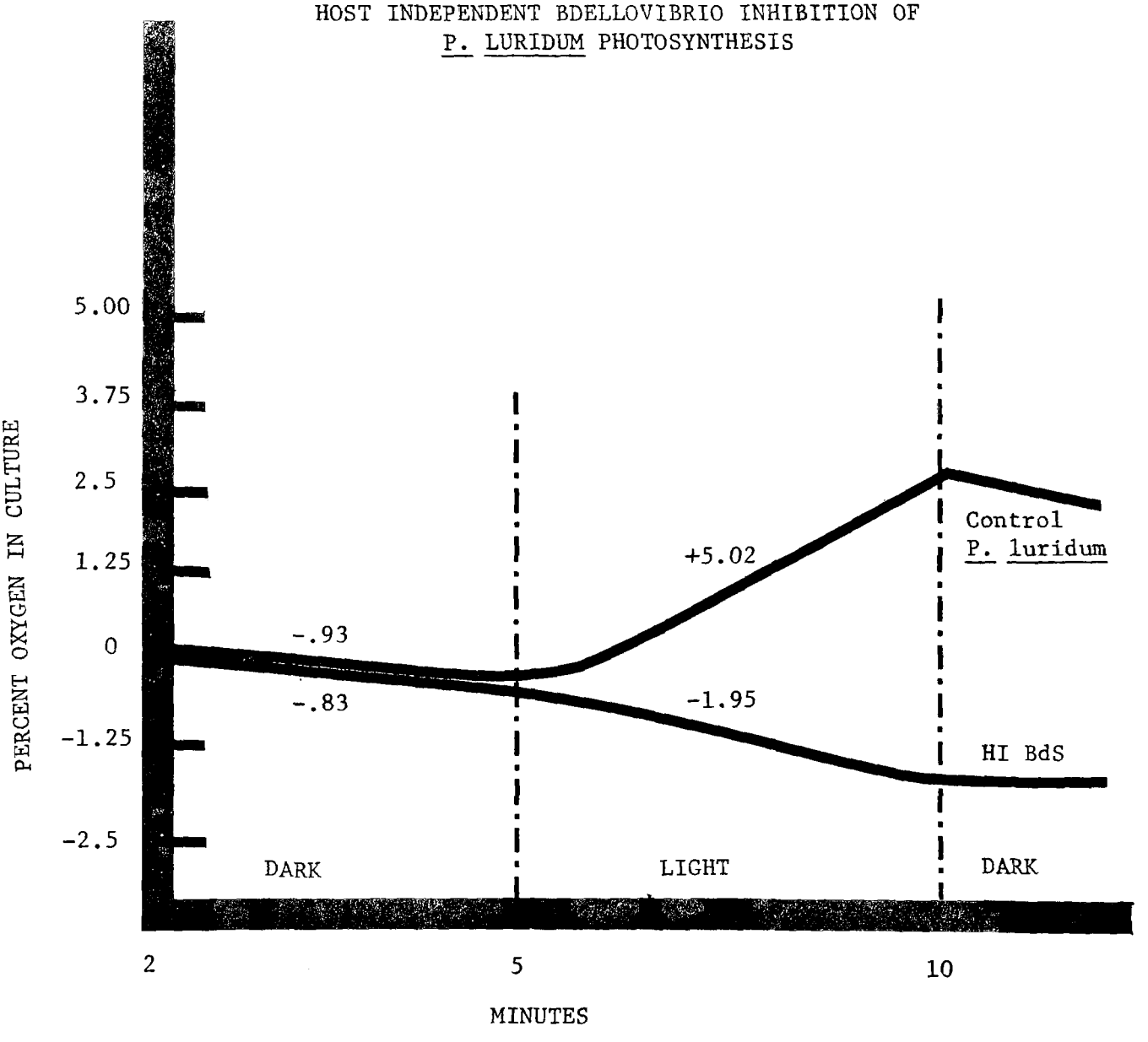
PROTEASE VS. PERCENT INHIBITION PHOTOSYNTHESIS



5

Figure 6 Curve showing the inhibition of P. luridum photosynthesis by host-independent B. bacteriovorus cell-free culture supernatant fluid at pH 8.5. Numbers refer to ul O_2/ug Chla/hr given off (+) or taken up (-). Note that similar to host-dependent bdellovibrio, the supernatant caused the phenomenon of photorespiration in the light. The curve represents the tracing obtained directly from a recorder connected to the Yellow Springs Instruments oxygen analyzer housed in a chamber designed by Burnham and Stetak (see Burnham, 1973).

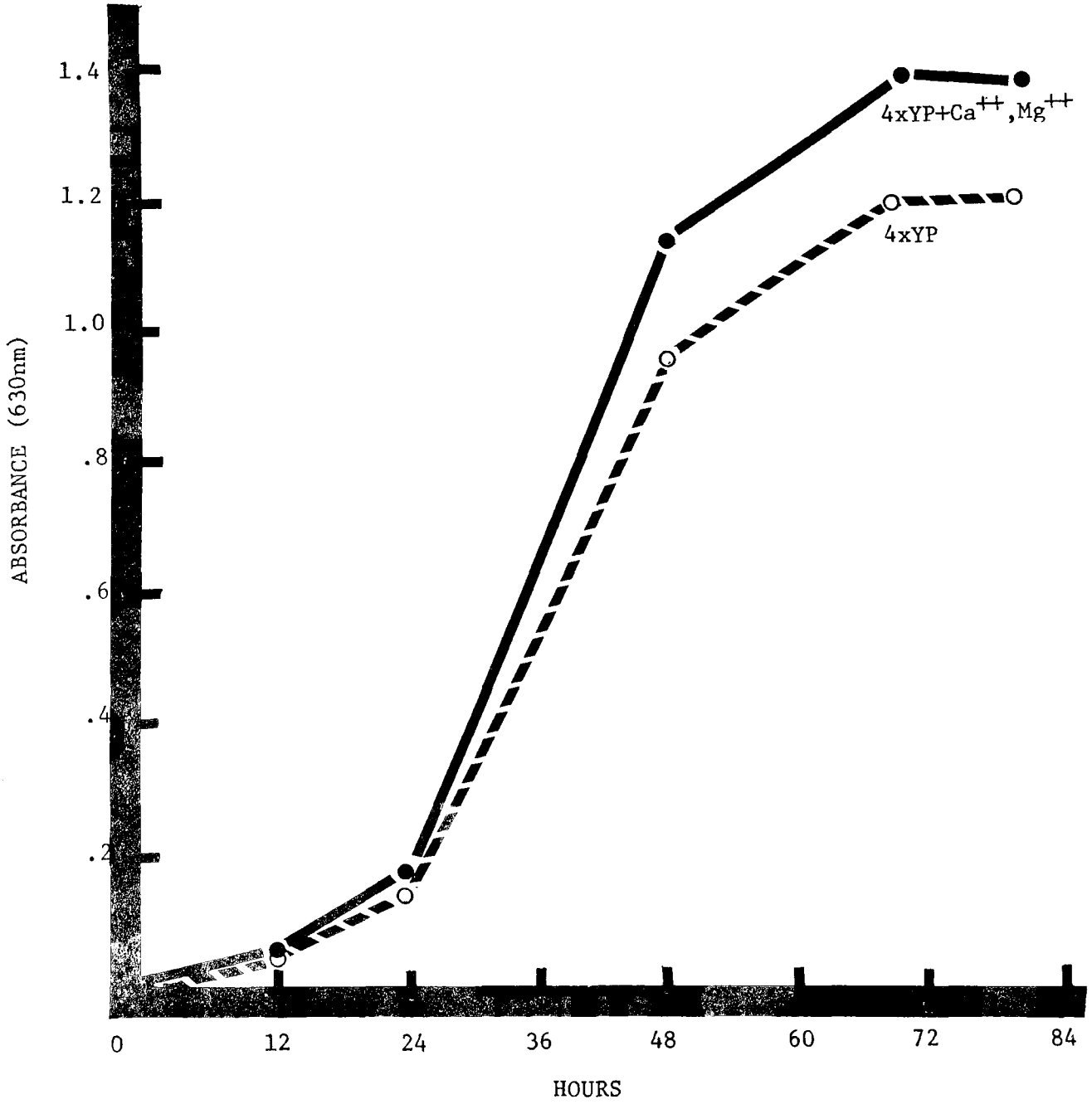
HOST INDEPENDENT BDELLOVIBRIO INHIBITION OF
P. LURIDUM PHOTOSYNTHESIS



6

Curve 7 A curve illustrating the growth of host-independent B. bacteriovorus 15143 in 4xYP and in 4xYP supplemented with 0.002 M. $MgCl_2$ and 0.002 M. $CaCl_2$. Cells were grown in 500 ml side arm flasks on a rotary shaker at 25 C and absorbance measured on a Coleman Junior 2A spectrophotometer.

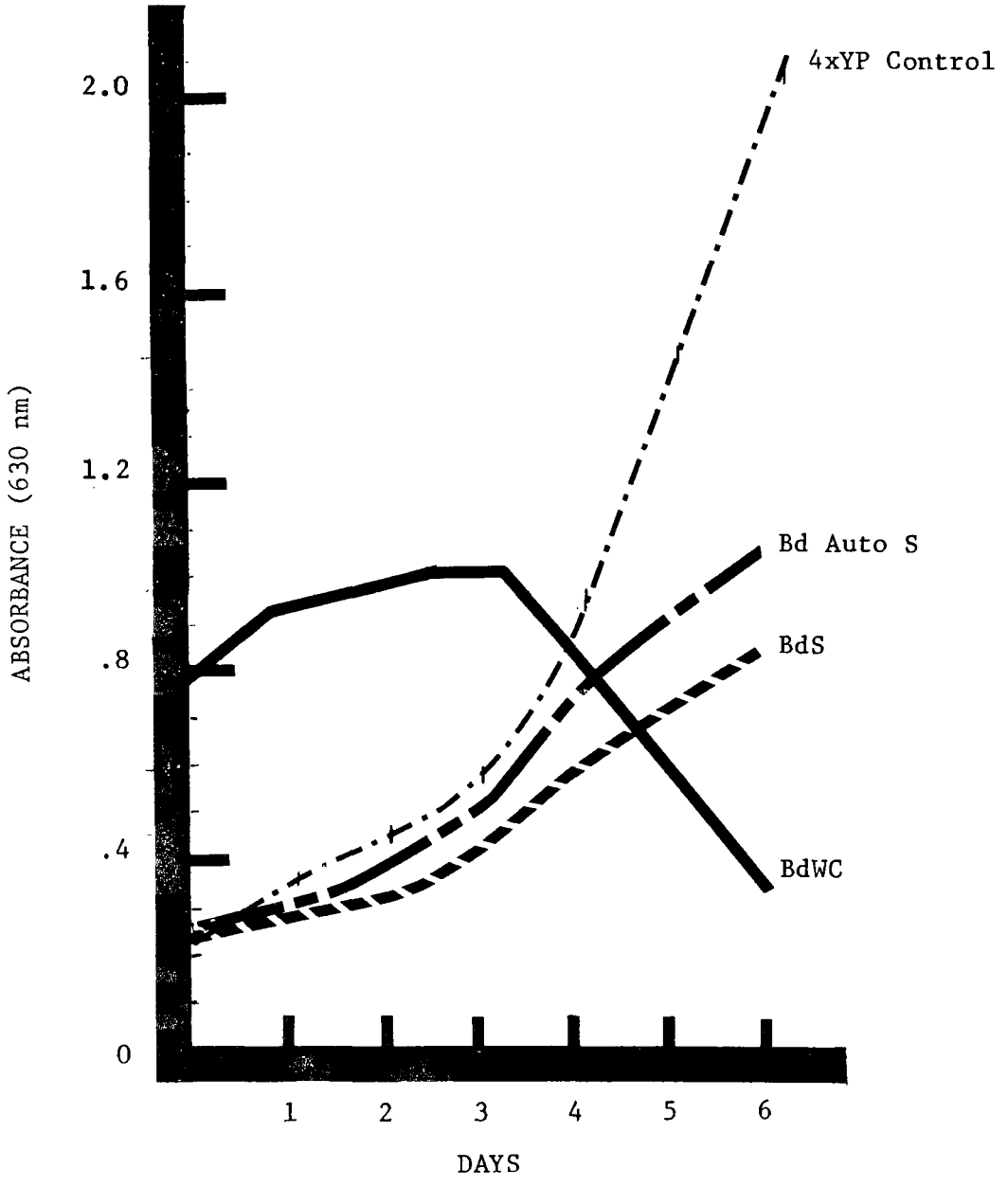
GROWTH CURVE FOR HOST INDEPENDENT MUTANT OF
BDELLOVIBRIO BACTERIOVORUS 15143
(1% INOCULUM)



7

Figure 8 A curve illustrating the effect of host-independent B. bacteriovorus 15143 and culture supernatant fractions on growth of P. luridum. Only whole cell bdellovibrios (BdWC) showed complete inhibition with lysis while the normal and autoclaved cell-free supernatant fluid (Bd S and Bd Auto S) showed about 50% inhibition of control levels. Oxygen inhibition showed similar results until pH was adjusted to 8.5 as indicated in Fig 11.

HOST INDEPENDENT BDELLOVIBRIO BACTERIOVORUS
INHIBITION OF PHORMIDIUM LURIDUM GROWTH

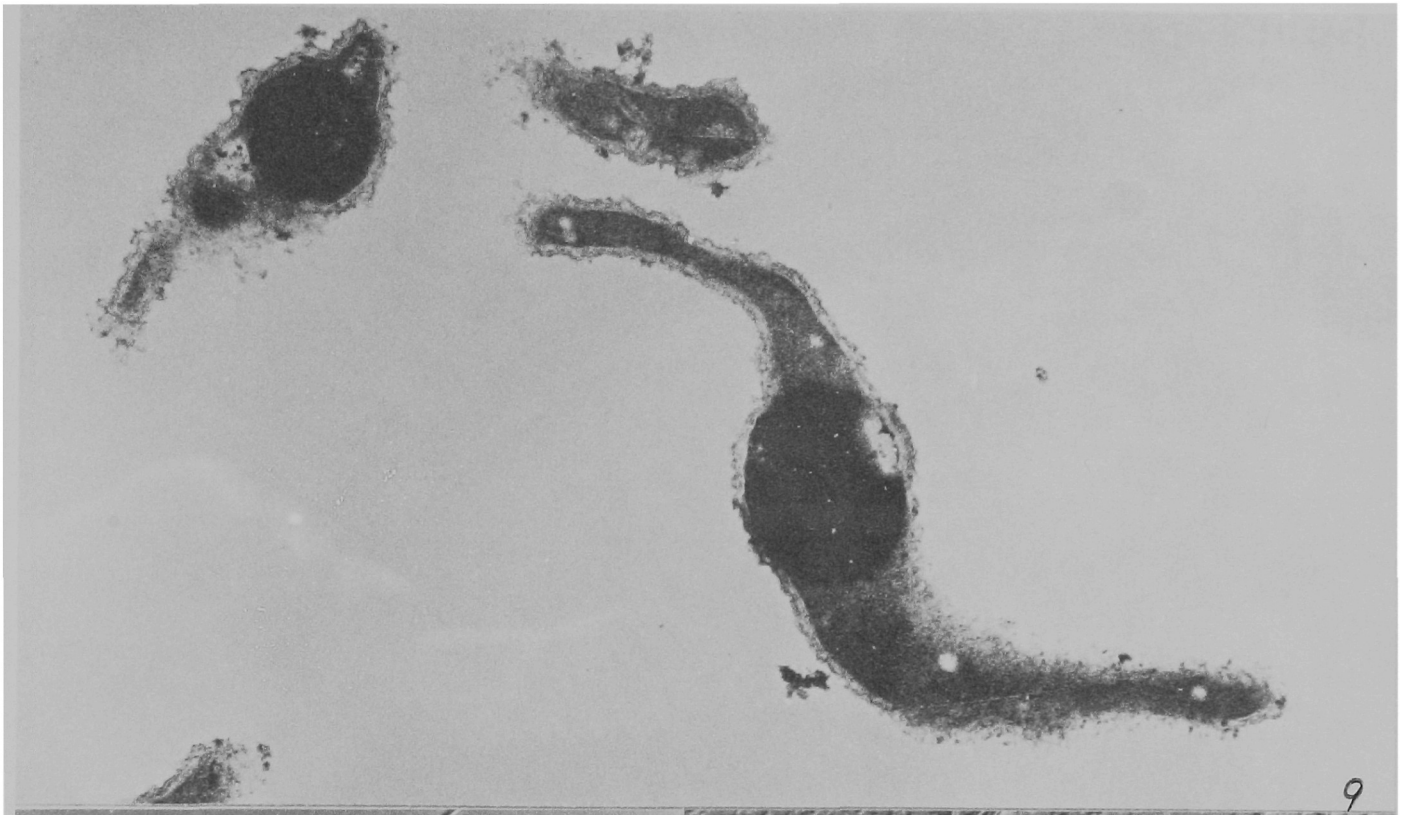


8

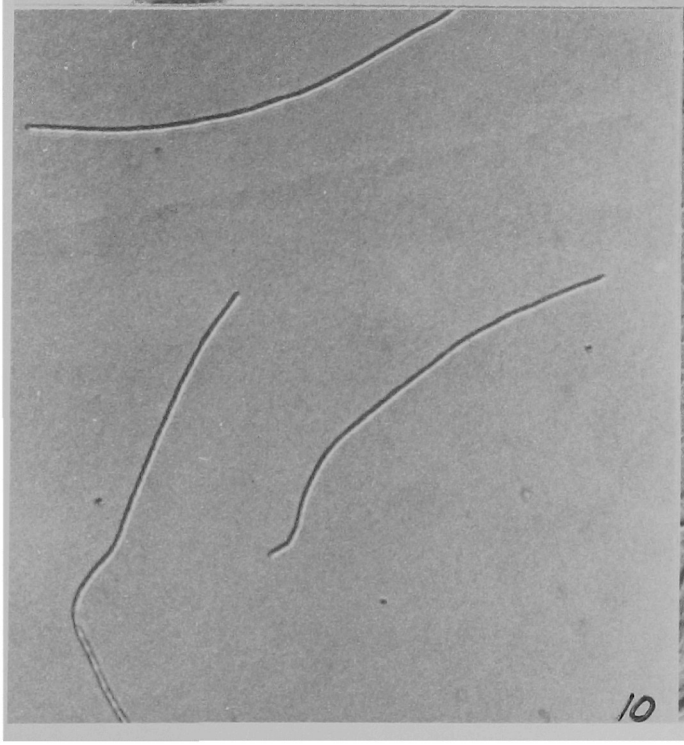
Figure 9 Electron microscopy (thin section) of host-independent B. bacteriovorus 15143 grown in 4xYP for 24 h. Cells are irregularly shaped containing many mesosomes and extremely large electron-dense structures thought to be polyphosphate bodies. Cell wall is normal gram-negative structure.

Figure 10. A micrograph of P. luridum inoculated on algae agar and examined after 96 h by Nomarski optics on an agar block (see Fig 3). The figure illustrates the slow growth that is characteristic of P. luridum when E. coli is absent.

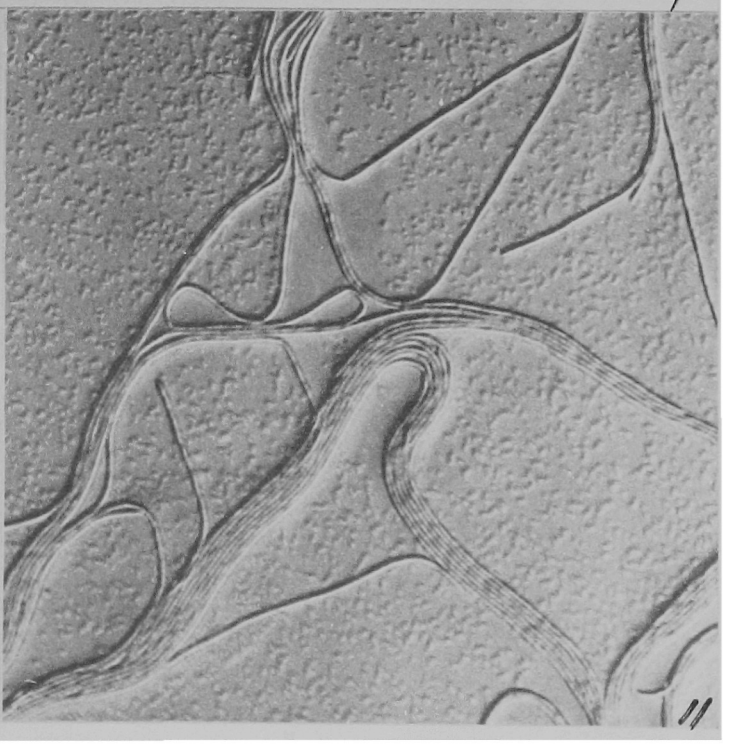
Figure 11 A micrograph of P. luridum inoculated with E. coli on an algae agar plate and directly examined after 96 h by Nomarski optics. These plates were inoculated at the same time as those in Fig 10 and at the same concentration.



9



10



11

Figure 12 This curve shows that this bacterial isolate has considerable inhibitory effect on the growth of P. luridum. The G_2 cells were grown in 4xYP and added 1:1 to the cyanobacterial culture. G_2 supernatant was prepared identically to the bdellovibrio supernatant (centrifugation and filtering through a 0.2 um pore filter). Control growth of P. luridum culture 1:1 with 4xYP is also illustrated.

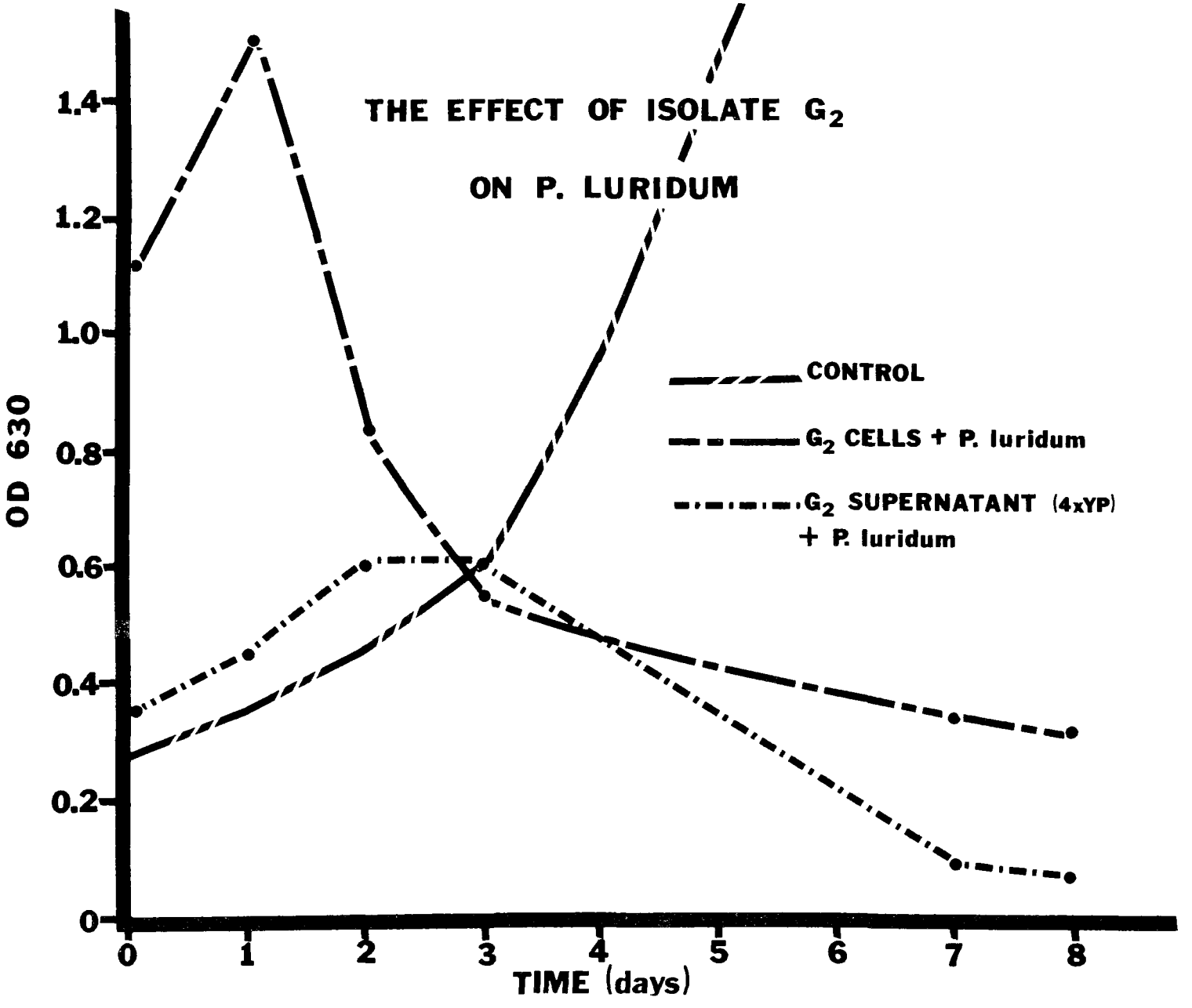

THE EFFECT OF ISOLATE G_2
ON P. LURIDUM
OD 630
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
7
8
TIME (days)
CONTROL
G_2 CELLS + P. luridum
G_2 SUPERNATANT (4xYP)
+ P. luridum

TABLE 1

Molecular Weight of Ps Inhibitor
(Amicon Thin Channel Filtration)

Test	Percent Recovery	Percent PS
Untreated	100	0*
100,000	57.9	15.4
50,000	63.2	0
30,000	94.7	0*
10,000	84.2	0*
1,000	10.5	77

*Respiration induced by light

TABLE 2

EFFECT OF PROTEASES ON PS INHIBITION

TEST	PERCENT PS
CONTROL	100
AUTO S	7
PRONASE - AS	50
TRYPSIN - AS	55

TABLE III. IDENTIFICATION OF ALTERNATE INHIBITORY BACTERIA

Test / Isolate #	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLV	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	NIT	OXID	MOTILITY	GRAM	SPORES	Possible Name
110	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	–	–	+	+	Bacillus
119	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	?
129	–	+	–	–	+	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	+	+	–	+	+	Bacillus
WP1	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	+	Bacillus
WP3a	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	?
WP3b	–	+	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	–	–	+	+	Bacillus
Ft.M.l, a	–	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+	+	+	+	Clostridium
Ft.M.l, b	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	Pseudomonas sp.
$SC3_2b$	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	?
F_2	–	+	–	–	+		–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	+	+	–	–	?
Greg's 2	–	+	–	–	–	–	–	+	+	+	+	+	–	+	–	+	–	–	+	+	+	+	+	–	–	?
Bact. 4	–	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–	?
(control) E. coli 15144	+	–	+	–	–	–	–	–	+	–	–	+	+	–	+ weak	+ weak	–	–	–	+	+	–	–	–	–	E. coli

Table IV

Effect of lyophilization of autoclaved bdellovibrio supernatant fractions on photosynthesis inhibition.

	%I*
Control	0
BdAS unfiltered	142.5
BdAS 50,000	111.4
BdAS 30,000	81.9
BdAS 10,000	93.3
BdAS 5,000	86.6
BdAS 1,000	79.4
BdAS 500	74.7

*%I is calculated as $[100 - \frac{\text{ul } O_2/\text{hr experimental}}{\text{ul } O_2/\text{hr control}} \times 100)]$

Table V

Effect of storage at -90C on the photosynthetic inhibitory properties of bdellovibrio supernatant.

Active Fraction	Storage Time	Media	$\%O_2$ Inhibition
BdS	0	YP	50.4
BdS	26 mo	YP	27.0
BdS	0	4xYP	132.0 (photorespiration)
BdS	14 mo	4xYP	117.0

$$\%I \text{ is calculated as } \left[100 - \frac{\text{ul } O_2/\text{hr experimental}}{\text{ul } O_2/\text{hr control}} \times 100\right]$$

STUDENTS WORKING ON THIS PROJECT

1. Mr. Donald C. Sun - obtained his Ph.D. doing his dissertation research on a project that related to the OWRR algal control matching grant. His dissertation title is "A Cytochemical and Physiological Study of Photosynthetic and Respiratory Electron Reactions in Phormidium luridum var. olivacea." A short summary is included below. For further data, see appendix article, Sun and Burnham, 1977.

Various cyanobacterial inhibitors and cyanobacteriocidal toxins produced by Bdellovibrio bacteriovorus (BdAS) were compared for their effect on Phormidium luridum, with regard to morphology, ultrastructure, photosynthesis, respiration, in vitro ferricyanide reduction and electron transport cytochemistry.

Electron microscopy reveals a similar cellular response to different cyanobacterial inhibitors, i.e., dichloromethylurea (DCMU); potassium cyanide (KCN); and BdAS. Initial morphological alterations involved the splitting of two closely paired photosynthetic membranes creating intrathylakoidal spaces. Prolonged incubation of P. luridum with KCN or BdAS caused disruption of the mucopeptide layer and leakage of cytoplasmic materials.

At 2000 fc, whole cells of P. luridum produced 405.4 ul O_2/mg chl a/hr, while fresh spheroplasts produced 326.7 ul O_2/mg chl a/hr, an efficiency of 80%. The respiration rate of spheroplasts (-84.5 ul O_2/mg chl a/hr) was actually higher than whole cells (-45 ul O_2/mg chl a/hr). The inhibitors, rotenone, ouabain, and amphotericin B did not affect either the photosynthetic or respiratory activities of P. luridum, whereas BdAS caused photorespiration in the light and a slight respiration inhibition in the dark.

The rate of ferricyanide reduction by whole cells of P. luridum was 126.6 uM/mg chl a/hr. Higher ferricyanide reduction rates were observed in polymyxin B-treated cells (451.2 uM/mg chl a/hr) and spheroplasts (331.2 uM/mg chl a/hr). The ferricyanide reduction rate was stimulated by O-phenanthroline and BdAS and decreased by DCMU, KCN, ouabain and rotenone.

Copper ferrocyanide electron cytochemistry was nonspecific except in cells treated with sodium lauryl sulfate. Deposition of 3, 3-diaminobenzidine (DAB) was seen in the intrathylakoidal space. Cytochemical localization, using tetranitroblue tetrazolium (TNBT), was not affected by KCN, DCMU, rotenone and O-phenanthroline, but was slightly inhibited by BdAS and severely inhibited by ouabain and polymyxin B. All reactions were tested under both light and dark conditions.

Cytochemical results showed that the photosynthetic lamellae of P. luridum also functioned as reactive sites for respiration. The intrathylakoidal space of P. luridum possessed cytochrome c oxidase activity similar to that localized in intracristal spaces of eukaryotic cells. Physiological and cytochemical data indicated that the inhibitory mechanism of BdAS in P. luridum was not identical to other inhibitors used in this study. Evidence suggests that BdAS interacted with the glycolytic pathway which caused an abnormal oxygen consumption in the presence of light.

2. Mr. Gregory Locher - Mr. Locher was just beginning his M. S. research project on the effect of bdellovibrio on attached algae when this project ended. This research will be continued in the Phase II matching grant for this project.

PERTINENT REFERENCES ORIGINATING FROM THIS GRANT PROGRAM

1. Burnham, J. C. and T. Stetak. 1972. Observations on the interaction of Bdellovibrio bacteriovorus with two species of blue-green algae. Abs. Ann. Mtg. Am. Soc. Microbiol. p. 33 (G18).

2. Burnham, J. C. 1973. Bacterial control of aquatic algae. Project Completion Report N. 414X. State of Ohio Water Resources Center, U. S. Department of Interior.

3. Burnham, J. C. and J. Robinson. 1973. The genus and species of Bdellovibrio. In N. Gibbons and R. E. Buchanan, eds. Bergey's Manual of Determinative Bacteriology, 8th Edition. In press.

4. Burnham, J. C., T. Stetak, and J. Boulger. 1973. An improved method of cell enumeration for filamentous algae and bacteria. J. Phycol. 9: 346-349.

5. Burnham, J. C., D. C. Sun and T. Stetak. 1974. Biological properties of a Bdellovibrio-produced inhibitor of blue-green algae. Abs. Ann. Mtg. Am. Soc. Microbiol. G 255.

6. Burnham, J. C. and D. C. Sun. 1975. Ultrastructural observations on the interaction of Bdellovibrio bacteriovorus with Phormidium luridum and Synechococcus sp. (cyanophyceae). J. Phycology. 13:203-208.

7. Burnham, J. C., T. Stetak, and G. Locher. 1976. Extracellular lysis of the blue-green algae Phormidium luridum var. olivacea. J. Phycol. 12:306-313.

8. Burnham, J. C., G. Locher and D. C. Sun. 1977. Photorespiration during thermally and bacterially induced stress in the cyanobacterium, Phormidium luridum. Abs. Ann. Mtg. Am. Soc. Microbiol. p. 241 (N74).

9. Sielicki, M. and J. C. Burnham. 1973. The effect of selenite on the physiological and morphological properties of the blue-green alga Phormidium luridum var. olivacea. J. Phycol. 9:509-514.

10. Sielicki, M. and J. C. Burnham. 1973. The effect of selenium on the physiology and structure of Phormidium luridum. Abs. Ann. Mtg. Am. Soc. Microbiol. G38.

11. Sun, D. C., G. Locher and J. C. Burnham. 1975. Comparison of photosynthetic inhibition in spheroplasts and whole cells of Phormidium luridum. Abs. Ann. Mtg. Am. Soc. Microbiol. p. I71.

12. Sun, D. C. and J. C. Burnham. 1977. A cytochemical study of photosynthetic and respiratory electron reactions in Phormidium luridum var. olivacea. Can. J. Microbiol. See Appendix of this report.

IN PREPARATION

Burnham, J. C. and G. Locher. 1977. Characterization of a cyanobacterial photosynthetic inhibitor produced by Bdellovibrio bacteriovorus.

Sun, D. C. and J. C. Burnham. 1977. A comparison of in vitro and in situ ferricyanide reduction reactions in whole cells and spheroplasts of Phormidium luridum var. olivacea.

APPENDIX

The following reports describing results and discussion of research from this OWRT Grant are included in this appendix:

1. Burnham, J. C., D. Sun and T. Stetak. 1974. Biological properties of a Bdellovibrio-produced inhibitor of blue-green algae.

2. Sun, D. C., G. Locher and J. C. Burnham. 1975. Comparison of photosynthetic inhibition in spheroplasts and whole cells of Phormidium luridum.

3. Burnham, J. C., G. Locher and D. C. Sun. 1976. An ultrastructural analysis of photosynthetic stress in the cyanobacteria, Phormidium luridum and Microcystis aeruginosa.

4. Burnham, J.C. and D. Sun. 1977. Electron microscope observations in the interactions of Bdellovibrio bacteriovorus with Phormidium luridum and Synechococcus sp. (Accepted by J. of Phycology).

5. Burnham, J. C. 1975. Bacterial Control of Aquatic Algae.

6. Sun, D., and J. C. Burnham. 1977. A cytochemical study of photosynthetic and respiratory electron reactions in Phormidium luridum var. olivacea.

Reprinted from *Abstracts of the Annual Meeting-1974*

G 255 Biological Properties of a Bdellovibrio-produced Inhibitor of Blue-green Algae. J.C. Burnham, D. SUN and T. STETAK, Dept. of Microbiol., Med. Col. of Ohio, Toledo, Ohio

Growth of Phormidium luridum and Microcystis aeruginosa cultures has been shown to be inhibited by equal volumes of heat treated (100°C/20 min.) cell free culture extracts of Bdellovibrio bacteriovorus 15143. Measurement of photosynthesis shows a 90 percent inhibition of O_2 evolution in 15 min. with 100 percent inhibition found after an hour of exposure to the inhibitor. Respiratory activity of the exposed blue-green algae is not affected. Ultrastructural observations show no effect of the inhibitor on the cell envelope but exposed algae exhibit poor cytoplasmic fixation, especially in the peripheral region. It appears that all inhibitory activity is reversible as removal of the extract allows return of photosynthetic ability and subsequent growth and division. The inhibitor is inactivated at a pH less than 7.0 and above 11.0. Vacuum dialysis indicates the size of the inhibitor to be less than 30,000 MW but all activity has been lost upon further purification by membrane dialysis or gel filtration. Treatment with various proteases show an average reduction of 50 percent inhibitory activity. This inhibitor appears to be algalstatic in aqueous environments and is effective in preventing the colonization of solid substrates by these blue-green algae.

I 71 Comparison of Photosynthetic Inhibition in Spheroplasts and Whole Cells of Phormidium luridum.
D. C. SUN*, G. LOCHER and J. C. BURNHAM, Dept. of Microbiol., Med. Col. of Ohio, Toledo, Ohio

Dichloromethylurea, potassium cyanide and a photosynthetic inhibitor produced by Bdellovibrio bacteriovorus ATCC 15143 are compared for their effect on whole cells and spheroplasts of Phormidium luridum, with regard to morphology, ultrastructure, photosynthetic O_2 evolution and in vivo ferricyanide reduction. At 2000 fc, P. luridum whole cells produced 7585 ul O_2/mg Chl a/hr while fresh spheroplasts produced 2965 ul O_2/mg Chl a/hr - an efficiency of 40 per cent. At 6000 fc, spheroplasts showed greater reduction of ferricyanide than whole cells; 1173 uM/mg Chl a/hr to 814 uM/mg Chl a/hr, respectively. Dark reactions showed 60 per cent less ferricyanide reduction. Both KCN and DCMU inhibited all oxygen evolution in whole cells and spheroplasts. Addition of KCN stimulated ferricyanide reduction over 54 per cent to 1806 uM/mg Chl a/ml. DCMU completely inhibited light dependent ferricyanide reduction, but had no effect on dark respiratory O_2 uptake or ferricyanide reduction. Addition of the bdellovibrio antialgal factor, a 100 C heat resistant, 10,000 MW substance secreted by viable cells, showed similar inhibitory activity as DCMU. This data suggests a similar site of action for the bdellovibrio factor, i.e., the inhibition of photosystem II in P. luridum.

I 113 An Ultrastructural Analysis of Photosynthetic Stress in the Cyanobacteria, Phormidium luridum and Microcystis aeruginosa. J.C. BURNHAM*, G. LOCHER and D.C. SUN, Dept. of Microbiology, Med. College of Ohio, Toledo, Ohio.

When 96 h grown cultures of Phormidium luridum and Microcystis aeruginosa are exposed to different photosynthetic inhibitors such as dichlorodimethylurea, potassium cyanide, sodium selenite and a heat-resistant-dependent or independent Bdellovibrio bacteriovrous ATCC 15143, these species show a remarkably uniform response in terms of their ultrastructural alteration. Observations of ultrastructural pathology were correlated with phase contrast microscopy and measurement of cell respiration and photosynthesis. P. luridum predominately showed gradual formation of large intrathylakoidal spaces and deterioration of the cell wall mucopeptide layer, finally resulting in residual membrane skeletons. In contrast, M. aeruginosa showed a more severe disaggregation of the thylakoid structure and little effect upon the mucopeptide layer of the cell wall, leaving residual cell wall skeletons. Evidence is accumulating that in P. luridum and M. aeruginosa the response to photosynthetic stress is the triggering of an algal autolytic system with the nature of the autolytic enzymes determining the algal pattern of ultrastructural demise.

ELECTRON MICROSCOPE OBSERVATIONS ON THE INTERACTION OF *BDELLOVIBRIO BACTERIOVORUS* WITH *PHORMIDIUM LURIDUM* AND *SYNECHOCOCCUS* SP.

Jeffrey C. Burnham and Donald Sun

Department of Microbiology

MEDICAL COLLEGE OF OHIO

Toledo, Ohio 43614

SUMMARY

The effects of Bdellovibrio bacteriovorus (Stolp and Starr) culture supernatants on Phormidium luridum var. olivacea Boresch and Synechococcus sp. were examined by transmission electron microscopy. Both normal (non-heat-treated) and heat-treated bdellovibrio supernatant caused the formation of intrathylakoidal vesicles in P. luridum in 24-48 h. This vesiculation increased until 96-129 h, when the P. luridum showed loss of the mucopeptide layer in the cell envelope and subsequently lysed. Similar treatment of Synechococcus sp. with the bdellovibrio supernatants showed a different ultrastructural pattern with the apparent dissolution of many of the photosynthetic membranes in the bluegreen cells. Myelin-like membraneous configurations were seen in some of these treated cells. The results suggest that an autolytic mechanism in P. luridum and Synechococcus sp. is stimulated by the bdellovibrio secretions.

Key index words: Bdellovibrio; lysis, Microcystis; Phormidium; Synechococcus; thylakoid; ultrastructure

INTRODUCTION

In a previous report, it was indicated that Bdellovibrio bacteriovorus was capable of causing the lysis of several species of bluegreen algae (3). Phase contrast microscopy indicated that morphological alterations in the bluegreen algae were noticeable several hours after exposure to either viable bdellovibrio cells, cell-free culture supernatant, or heat-treated supernatant. Accompanying these initial morphological changes was a loss of photosynthetic ability (3). Continued observation showed an intracellular deterioration of the bluegreen algae and a general breakup of the trichome organization.

Although several different species of bacteria have been described to lyse bluegreen algae (4, 5, 11, 13, 18), little attention has been paid to the morphological and ultrastructural changes in these bluegreen algal species during their degradation. This paper describes the sequences of ultrastructural events in this bdellovibrio/algal interaction in order to: (i) determine if the photosynthetic system is directly affected; (ii) to provide additional information on the comparative breakdown of P. luridum and Synechococcus sp. and (iii) develop data for the evaluation of such a system for the biological control of algae.

MATERIALS AND METHODS

B. bacteriovorus, American Type Culture Collection (ATCC), strain 15143, was routinely maintained on host bacterium Escherichia coli, ATCC strain 15144. P. luridum, var. olivacea Boresch (No. 426) was obtained from the Culture Colection of Algae at Indiana University.

The Synechococcus sp. was obtained from ATCC as Microcystis aeruginosa (Kutz) Lemmerman, strain 18800. Because of the uncertainty of the past history of this strain and its identity as a Synechococcus sp. by Stanier et al. (16), we choose to use the Synechococcus designation.

The algae were grown in 500 ml Erlenmeyer flasks placed on a rotary shaker at 300 ft-c and at 30 C. P. luridum were grown axenically in Difco algae broth (designated AB) and Synechococcus sp. axenically in Hughes 11 medium (6). A 20% inoculum was allowed to develop for 96 h before being mixed with bdellovibrios.

B. bacteriovorus was maintained as previously described (2, 3). In preparation for addition of the bdellovibrios to the algal cultures, a 5% inoculum of B. bacteriovorus was placed in a 24 h culture of E. coli grown in a 1.2% yeast extract (Difco) snd 0.24% peptone (Difco) medium (designated 4xYP), and allowed to develop 24 h at 30 C on a rotary shaker. Light microscopic observations confirmed maturation of the bdellovibrio culture as indicated by absence of host E. coli.

Bluegreen algal cultures were interacted with equal volumes of bdellovibrio culture or cell-free culture supernatant as previously described (3). Mixed cultures were incubated at 25 C on a rotary shaker under continuous 300 ft-c illumination. Heat treatment of cell-free bdellovibrio supernatant was carried out by autoclaving it for 20 minutes (3). Sampling for electron microscopy was correlated with phase contrast microscopic observations (3).

Specimens for examination by electron microscopy were immediately suspended 1:1 in 4% glutaraldehyde (Polysciences, Inc., Warrington, Pa.) in Ryter-Kellenberger buffer (7) and centrifuged for 10 m at 4000 RPM. The pellet was resuspended in glutaraldehyde and processed by standard bacterial fixation procedures (2). Control cells of P. luridum were always fixed and embedded in parallel with test specimens. Specimens were sectioned with a diamond knife on an LKB Ultratome III, and placed directly on 300 mesh copper grids and poststained with 2% uranyl acetate and Reynold's lead citrate stain (12). Samples were examined and photographed with a Philips EM300 electron microscope operating at 60 kv.

To prepare lysozyme-treated cells, egg white lysozyme in 4xYP medium (Sigma Chemical) was added to a 96 h culture of P. luridum to a final concnetration of 0.05%. Sampling and preparation of these specimens for electron microscopy was as described above for the bdellovibrio/bluegreen algal interaction.

RESULTS

Because the primary effect of the bdellovibrios is to distort the normal cellular organization of the bluegreen algae (3), it is necessary initially to illustrate the appearance of the P. luridum control cells. Figure 1 shows a longitudinal section through the trichome, illustrating the overall cytoplasmic compactness in the organization of the individual bluegreen cells. These cells often contain cyanophycin or structured granules (sg) (8). The photosynthetic thylakoids are arranged in concentric double membranes, each separated from the others by dense cytoplasm. Measurement of distance from one side of the normal thylakoid membrane pair to the other yields a mean of 210Å and only rarely was seen to form small intramembraneous vesiculations in control preparations. With the modified Ryter-Kellenberger fixation employed, a fibrous nucleoplasm is often visible in the central region of the cell.

The multilayered gram-negative type of cell wall is similar to that described for other bluegreen algae (8) (also see Fig 6).

When viable cells of B. bacteriovorus are introduced to a P. luridum culture as described in MATERIALS AND METHODS, it takes between 4 and 6 hours of interaction for definite signs of granulation to appear under phase contrast microscopic examination (3).

It takes between 16 and 24 h after exposure for the first appearance of ultrastructural pathology. Figure 2 shows one P. luridum with early splitting between the thylakoid membrane pair, while the other cell shows a more advanced separation. It should also be noted that the cell wall of the P. luridum, particularly the mucopeptide layer, is not observably affected this early after exposure to the bdellovibrios. This intrathylakoidal vesiculation continues as exposure is prolonged (48 h), as shown by Figs 3 and 4. The photosynthetic membrane can be seen to border each of these vesicles. There

is some question as to the content of the larger vacuolated areas, as in Figs 3 and 4 there appears in these cells to be a semi-transparent granular matrix that is not the same density as the epon embedding medium. The pathological distortion in P. luridum can be extreme as shown in Fig 4, with several regions of the thylakoid reorganizing into membrane-bound structures. Extended exposure of up to 72 h did not show removal of the dense mucopeptide layer in the cell wall. Only after 4 to 5 days' exposure did total cell distortion become widespread (see Fig 5).

We initially believed that the bdellovibrio lytic factor was causing a loss of permeability control by the bluegreen cell envelope, resulting in an influx of the hypotonic medium into the intrathylakoidal region. We exposed P. luridum to 0.05% lysozyme in 4xYP and periodically observed these cells by electron microscopy. Figure 6 details the laminated nature of the wall and emphasizes the thickness of the mucopeptide layer (arrow). Figures 7 and 8 illustrate 96 h and 48 h lysozyme-treated P. luridum, respectively. As previously reported, the primary action of lysozyme is the conversion of the cells to spheroplasts (1) with the loss of the 10 nm dense layer of mucopeptide in the cell envelope (9) (see Fig 7). Figure 7, in comparison with Fig 6, indicates that upon the loss of the mucopeptide layer, the overlying lipopolysaccharide layer closed up the resultant space, thereby reducing the total thickness of the cell envelope by approximately 39%. Figure 8 illustrates that although the lysozyme treatment conditions were identical to those with the bdellovibrio supernatant, the photosynthetic membrane system in the lysozyme-treated bluegreen cells generally did not separate and form intrathylakoidal spaces (Fig 8). The lysozyme treatment did result in the loss of the majority of the algal cytoplasm.

When the bdellovibrio cell-free supernatant was autoclaved, all proteolytic activity was destroyed as measured by the Azocoll test (3). When P. luridum was exposed to this supernatant for 48 h, intrathylakoidal vesiculation could be seen with the formation of protoplast-like bodies. The intramembranous spaces never developed to the overwhelming extent caused by viable bdellovibrios or by non-heat-treated culture supernatant.

Two structural consequences of incubating P. luridum in heat-treated supernatant were the apparent denser staining of the mucopeptide layer and the formation of localized lesions (non-staining areas) in this mucopeptide layer after 48 h to 96 h of interaction. If P. luridum was exposed over 96 h to the heat-treated supernatant, the trichomes eventually lysed, with the cells showing loss of cytoplasm or disruption of cell wall and photosynthetic membranes (Fig 10).

Cells of the axenically grown non-filamentous bluegreen alga Synechococcus sp. were also tested with the heat-treated bdellovibrio supernatant. Figure 11 shows a representative control cell, while Figs 12 and 13 illustrate the pattern of breakdown induced by the autoclaved bdellovibrio supernatant. The structural effects were considerably distinct from those shown by P. luridum. The photosynthetic thylakoids appeared to gradually disappear upon exposure to the bdellovibrio supernatant. Membranes often appeared to be reorganized within the cytoplasm as abnormal myelin-like figures (Fig 18). Intrathylakoidal vesiculation as seen in P. luridum was not observed in Synechococcus sp. Continued incubation of the Synechococcus cells for 96 h or more usually resulted in the formation of cell envelope skeletons. The relatively intact mucopeptide layer indicated that little or no lysozyme-like activity was present, either from the heat-treated supernatant, or from autolytic processes within the Synechococcus sp. cells.

DISCUSSION

The early morphological changes observed by phase contrast microscopy (3) when P. luridum cells are exposed to bdellovibrios were not distinguishable by our electron microscopic techniques. We suspect that this early granule formation is related to an aggregation of the photosynthetic pigments. This would correlate with our earlier observations (3) which showed a rapid loss of photosynthetic ability when the algae were exposed to either heat-treated or normal bdellovibrio secretions.

Intrathylakoidal vesiculation in bluegreen algae is not a new observation (8, 16). These two reports (8, 16) do not describe the photosynthetic ability of their bluegreen algal cells with the intrathylakoidal spaces, but our data (3) shows that P. luridum cells with these spaces had lost their photosynthetic ability, and were in the process of dying.

The substance produced by the bdellovibrios appears to be quite specific in action as evidenced by both its lack of effect on cell respiratory processes and its inhibition of the oxygen evolving photosynthetic system (3). Our testing (3) showed lysozyme (0.05%) exposure for 4 h inhibited both respiration and photosynthetic oxygen evolution in P. luridum. Ultrastructural evidence indicates that the cytoplasm was destroyed when P. luridum cells were suspended in lysozyme in the absence of isotonic conditions. This in all probability resulted in the killing of the cell, as Biggins (1) and Lindsey et al. (7) found osmotic buffering necessary to protect cell functions. Lysozyme treatment caused the formation of very few intrathylakoidal vacuoles in our preparations of P. luridum, even upon extended (96 h) incubation. These were significantly fewer than with the bdellovibrio substance. Lindsey, et al. (9) showed some "dilation" of the thylakoids of Anacystis nidulans with lysozyme-treated cells, but no large intrathylakoidal vesiculation. The

results with lysozyme suggest that the intrathylakoidal vesiculation in P. luridum is not due to the influx of hypotonic medium. The vesiculation may be due to an autolytic process triggered by the bdellovibrio secretions. Further evidence suggesting that there is such an autolytic process in P. luridum is provided by: (i) exposure of this bluegreen algae to selenium (15). When P. luridum was exposed to lethal concentrations of sodium selenite, photosynthesis was stopped (15) and similar intrathylakoidal vacuoles were formed (14) preceding lysis of the cells; (ii) the fact that the bdellovibrio supernatant is equally effective following autoclaving, which removes its measurableprotease activity; and (iii) the apparent digestion of the photosynthetic membrane of Synechococcus sp. upon exposure to the bdellovibrio supernatant---a result not related to any of structural events in the destruction of P. luridum, but yet stimulated by the same substance. We would expect that if the vesiculation were not autolytic, it would also have occurred in the Synechococcus sp.

The data accumulated to date suggests that the primary result of this bdellovibrio/bluegreen algal interaction to be a related sequence of: (i) inhibition of photosynthesis (3), coupled with (ii) a stimulation of an autolytic process. These results indicate this heat-resistant bdellovibrio-secreted material may hold potential as an effective algal control agent. It appears not to be species specific and may be an effective catalyst in causing the self-destruction of bluegreen algal cells.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to Mr. Tom Stetak and Mr. Gregory Locher for their assistance with the electron microscopy for this project.

This investigation was supported, in part, by a grant from the Toledo Edison Corporation, Toledo, Ohio; an Ohio Water Resources Grant (#B-051-Ohio), and a research support grant from the National Institutes of Health (#5-501-RRp-5700-02).

REFERENCES

1. Biggins, J. 1967. Preparation of metabolically-active protoplasts and particle preparations from the blue-green alga, Phormidium luridum. Plant Physiol. 1442-46.

2. Burnham, J. C., Hashimoto, T. and Conti, S. F. 1968. Electron microscopic observations on the penetration of Bdellovibrio bacteriovorus into gram-negative bacterial hosts. J. Bacteriol. 96:1366-81.

3. Burnham, J. C., Stetak, T. and Locher, G. 1975. Extracellular lysis of the blue-green alga Phormidium luridum var. olivacea. J. Phycol. 12:306-13.

4. Daft, J. J. and Stewart, W. D. P. 1971. Bacterial pathogens of fresh water blue-green algae. New Phytol. 70:819-29.

5. Granhall, U. and Berg, B. 1972. Antimicrobial effects of Cellvibrio on blue-green algae. Arch. Mikrobiol. 84:234-42.

6. Hughes, E. O., Gorham, P. R. and Zehnder, A. 1958. Toxicity of an unialgal culture of Microcystis aeruginosa. Can. J. Microbiol. 4:225-36.

7. Kellenberger, E., Ryter, A. and Sechaud, J. 1958. Electron microscopic study of DNA-containing plasma. II. Vegetative and mature phage DNA as compared to normal bacterial nucleoids in different physiological states. J. Biophys. Biochem. Cytol. 4:671-78.

8. Lang, N. J. 1968. The fine structure of the blue-green algae. Ann. Rev. Microbiol. 22:15-46.

9. Lindsey, J. K., Vance, B. D., Keeter, J. S. and Scholes, V. E. 1971. Spheroplast formation and associated ultrastructural changes in a synchronous culture of Anacystis nidulans treated with lysozyme. J. Phycol. 7:65-71.

10. Padan, E. and Shilo, M. 1972. Cyanophages-viruses attacking blue-green algae. Bact. Rev. 37:343-70.

11. Reim, R. L., Shane, M. S. and Cannon, R. E. 1974. The characterization of a bacillus capable of blue-green bacteriocidal activity. Can. J. Microbiol. 20:981-86.

12. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 17:208-12.

13. Shilo, M. 1970. Lysis of blue-green algae by Myxobacter. J. Bact. 104:453-61.

14. Sielicki, M. 1972. The effect of selenium on the ultrastructural and physiological properties of the blue-green alga, Phormidium luridum var. olivacea. Masters Thesis, University of Toledi, Toledo, Ohio. P. 118.

15. Sielicki, M. and Burnham, J. C. 1973. The effect of selenite on the physiological and morphological properties of the blue-green alga Phormidium luridum var. olivacea. J. Phycol. 9:509-14.

16. Smith, R. V. and Peat, A. 1967. Growth and gas vacuole development in vegetative cells of Anabaena flos-aquae. Arch. Mikrobiol. 58:117-26.

17. Stanier, R. Y., Kunisawa, R., Mandel, M., and Cohen-Bazire, G. 1971. Purification and properties of unicellular bluegreen algae (order Chroococcales). Bact. Rev. 35:171-205.

18. Stewart, J. R. and Brown, R. M. 1969. Cytophaga that kills or lyses algae. Science. 164:1523-4.

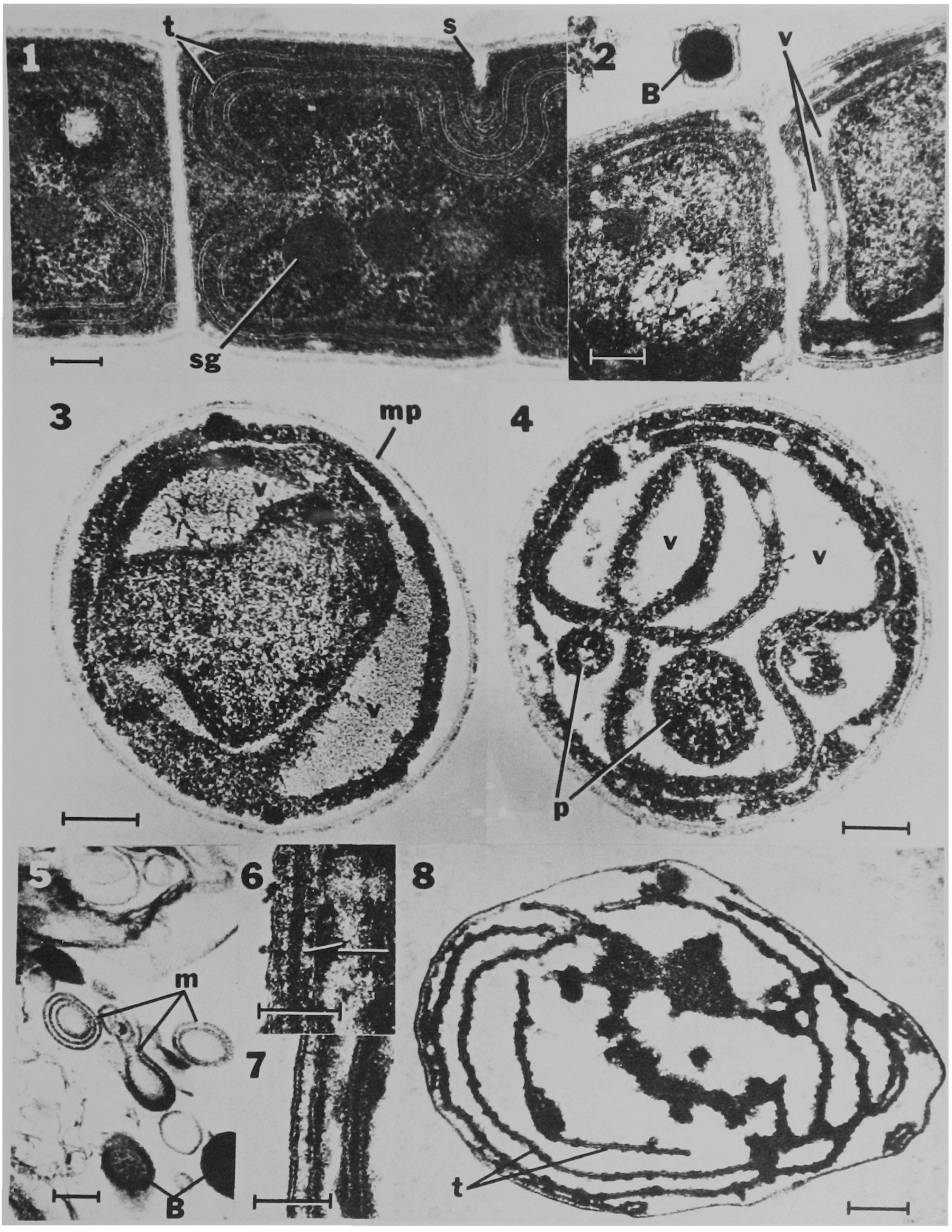
(PLATE I)

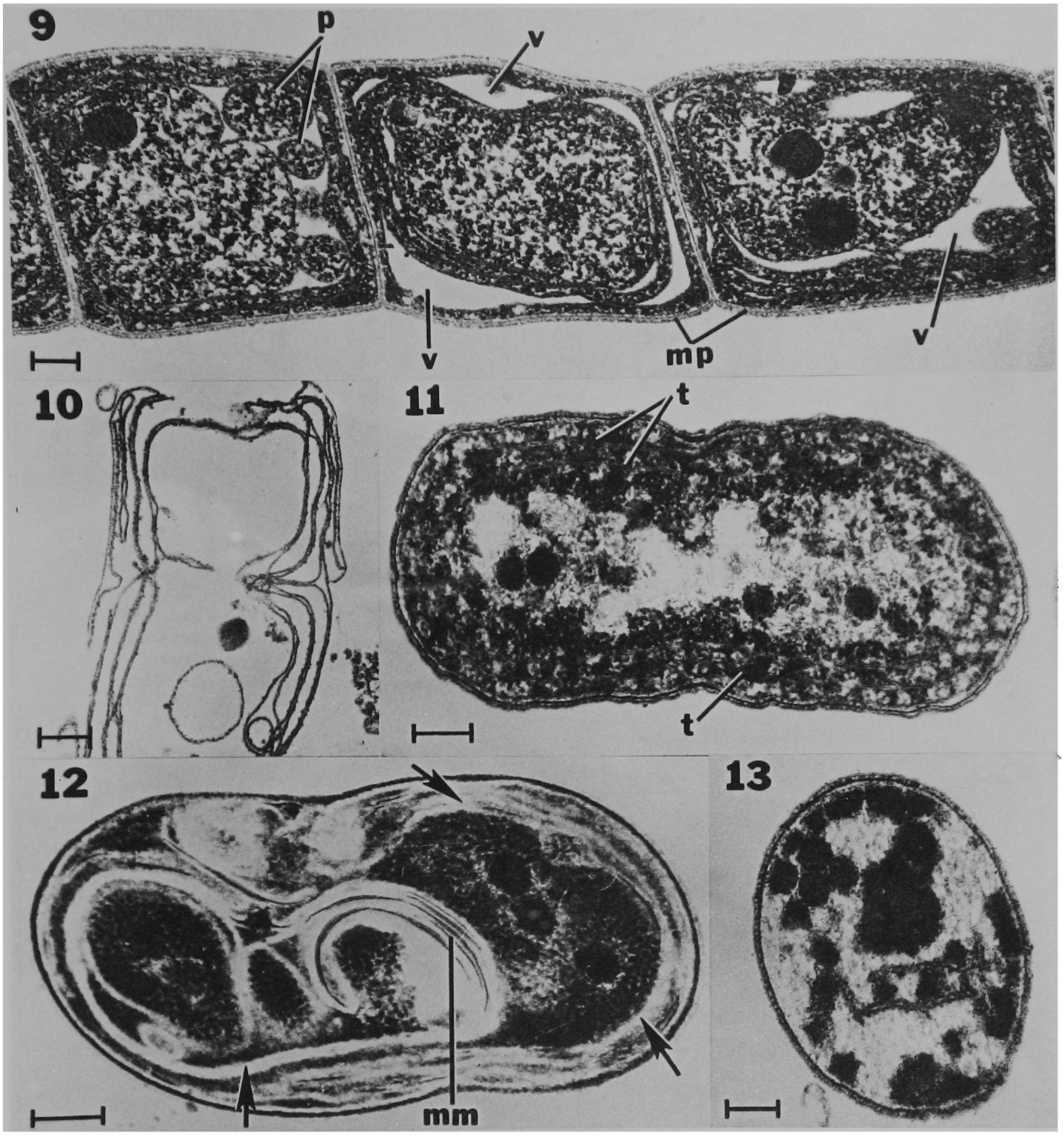
LEGEND FOR FIGURES

Fig. 1. A representative segment of a control P. luridum trichome axenically grown in 1:1 AB/YP for 96 h; note the uniform integrity of the double membrane thylakoids (t) peripherally and concentrically located in the cytoplasm; also the structured granules (sg) and the developing centripetal septum (s). The horizontal marker indicates 0.2 um on all micrographs. Fig 2. P. luridum after 24 h exposure to viable bdellovibrios showing the early stages of intrathylakoidal vesiculation (v). A cross section of the bdellovibrio cells (B) can be seen in this section. Fig. 3. P. luridum mixed with cell-free bdellovibrio culture supernatant for 48 h; note both the intermediate sized vesicles (v) which appear to contain a grainy material and the mucopeptide layer (mp) of the cell envelope which still appears to be intact. Fig 4. P. luridum after 48 h exposure to bdellovibrio supernatant; note that the intrathylakoidal vesicles (v) now occupy a large part of the cell space; also note the protoplast-like bodies (p) formed by enclosure of the cytoplasm by a photosynthetic membrane. Fig. 5. Detail of the photosynthetic membranes (m) and bluegreen cellular debris remaining after P. luridum was exposed to viable bdellovibrios for 5 days; also note two bdellovibrios (B). Fig 6. An enlarged section of a control P. luridum cell wall illustrating the mucopeptide layer (arrow) of 10 to 15 nm thickness. Fig 7. Detail of the P. luridum cell wall following 96 h exposure to 0.05% lysozyme; note the loss of the mucopeptide layer visible in Fig 6. Fig 8. Whole cell of P. luridum after 48 h treatment with 0.05% lysozyme; note the double membrane thylakoids (t) are generally intact and that the mucopeptide layer in the cell envelope has been removed.

(PLATE II)

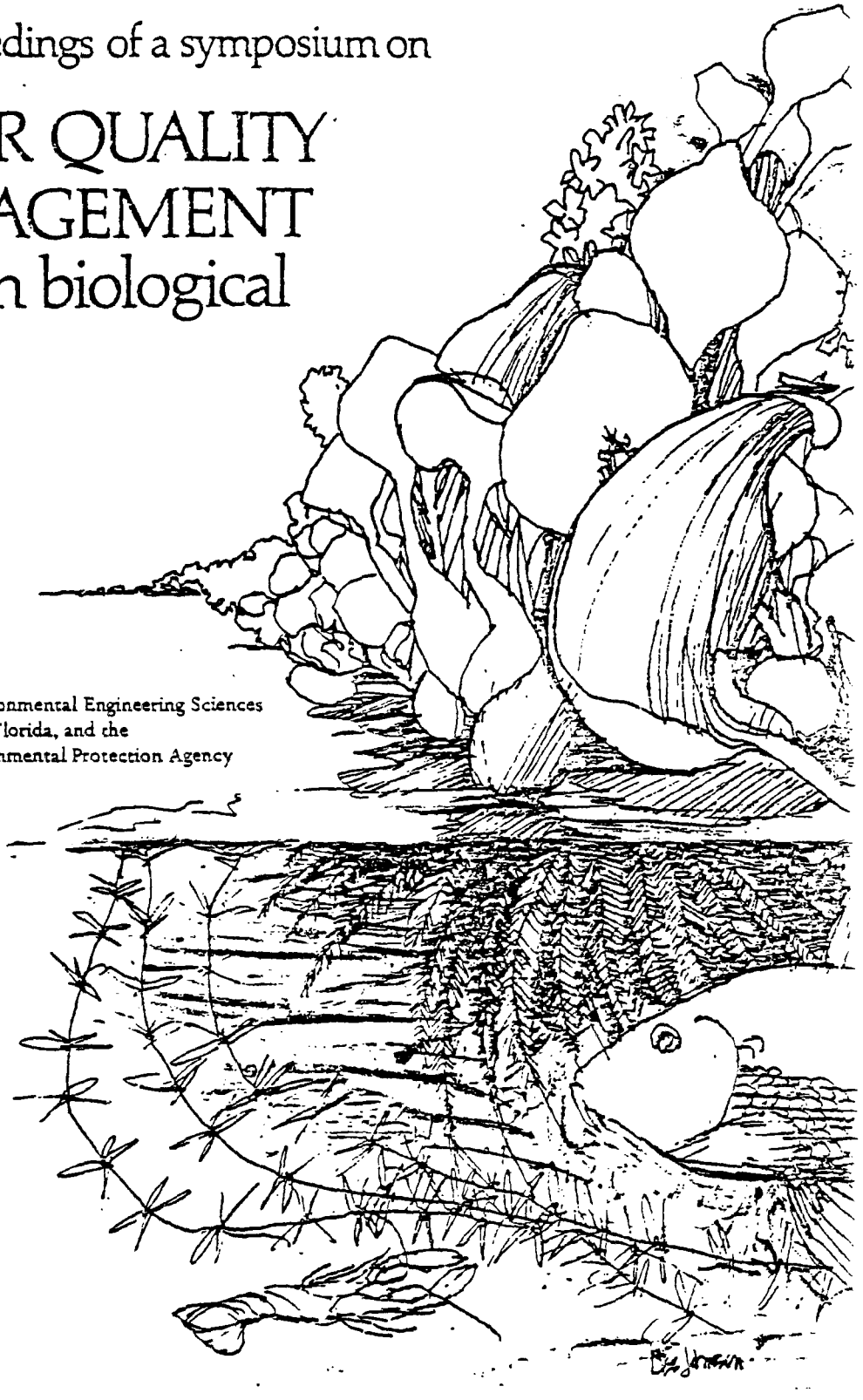
Fig 9. A longitudinally sectioned P. luridum cell after 48 h exposure to autoclaved bdellovibrio supernatant showing intrathylakoidal vesiculation (v) and protoplast-like bodies (p); note that the mucopeptide layer is still present. The horizontal line indicates 0.2 um on all micrographs. Fig 10. A lysed P. luridum after 96 h exposure to the autoclaved supernatant fluid; note that the lysis with heat-treated uspernatant yields a membraneous skeleton of single or dividing cells remaining from the disintegrated trichomes; also note that the mucopeptide layer has been removed. Fig 11. A control cell of Synechococcus sp. grown in Hughes 11 medium for 96 h.; note the intact configuration of the double membrane thylakoids (t). Fig. 12. Synechococcus sp. exposed to autoclaved cell-free bdellovibrio culture supernatant for 48 h; note that the photosynthetic thylakoids no longer are seen as stained unit membranes but instead appear as electron transparent spaces (arrows); note the myelin-like membrane pattern (mm) seen near the center of this cell. Fig 13. Synechococcus sp. after 96 h exposure to the autoclaved supernatant illustrating a more advanced loss of membranes with a concommitant loss of cytoplasmic integrity in spite of continued presence of a mucopeptide layer.


1
t
s
sg
2
B
v
3
mp
v
v
4
v
v
p
5
m
B
6
7
8
t


9
p
v
v
mp
v
10
11
t
t
12
mm
13

The proceedings of a symposium on

WATER QUALITY MANAGEMENT through biological control



January, 1975

Sponsored by the
Department of Environmental Engineering Sciences
at the University of Florida, and the
United States Environmental Protection Agency

The proceedings
of a symposium on

WATER QUALITY MANAGEMENT

through biological control

Held January 23-30, 1975
at the University of Florida

Edited by
Patrick L. Brezonik
Jackson L. Fox

Report No.
ENV-07-75-1

SPONSORED BY:
Department of Environmental
Engineering Sciences,
University of Florida
Gainesville, Florida
and the
National Eutrophication
Research Program,
Environmental Protection
Agency
Corallis Oregon

Department of Environmental Engineering Sciences
University of Florida
Gainesville, Fla. 32611

120

BACTERIAL CONTROL OF AQUATIC ALGAE

JEFFREY C. BURNHAM
Department of Microbiology
Medical College of Ohio
Toledo, Ohio 43614

The concept of microbiological control of pests has been recognized for many years. A principal advantage of the natural control system is that introduction of non-specific toxic chemicals into the environment is avoided. Basic ecological research and succeeding field experiments have since proven the value of microorganisms in control of unwanted populations. The use of a virus to control rabbit (Fenner 1959) and insect (Rivers 1964; Shea 1971) populations has been demonstrated. In recent years, viral control of algal populations (Brown 1972; Safferman and Morris 1964, 1967) has been partially successful. This kind of approach, of artificially introducing a natural control agent for an undesired host, provided the stimulus for investigating bacterial parasitism of algal populations.

BACTERIA AND ALGAL LYSIS

The interactions between bacteria and algae that result in algal lysis include the lysis of both unicellular and filamentous blue-green algae by a *Myxobacter* species (Shilo 1970), and by a *Cytophaga* species (Stewart and Brown 1969), and the lysis of various higher algae by members of the genus *Pseudomonas* (Mitchell 1972). Daft and Stewart (1971) have also reported myxobacteria that lyse blue-green algae. By plaque assay they demonstrated that one bacterial cell can cause algal lysis. They demonstrated that intact bacterial cells were necessary for inhibition of the algae; cell filtrates had no effect on the algal cells. These authors also reported some success in limited field trials using myxobacteria to control *Microcystis aeruginosa* colonies (Fogg *et al.* 1973). Lysis in all of these cases has been caused by an exocellular enzyme that functions only upon solid medium. Until lysis of *Phormidium* by bdellovibrios in liquid systems was reported (Burnham and Stetak 1972), all algal lytic processes needed a solid substrate.

Vibrios and related bacteria have been shown to lyse algae. A vibrio was reported to attack several species of the green alga *Chlorella* by attaching to the cells and then lysing them (Mamkaeva 1966; Starr and Seidler 1971). The mechanism for this activity was not resolved. Granhall and Berg (1972) also described an antibiotic substance produced by the genus *Cellvibrio* that is capable of lysing vegetative cells of the blue-green alga, *Anabaena inaequalis*. It is particularly interesting that a bacterial protease had no effect on the algicide activity nor did boiling for 15 min. While pepsin had no effect, papain was two-thirds inhibitory. Molecular weight determinations by filtration indicate the weight of the inhibitor was between 1,000 and 10,000. The activity of this substance is apparently dependent on algal growth as darkness inhibited its lytic effect. This case differs from *Myxobacter* and *Cytophaga* lysis in its occurrence in liquid systems, and the authors consequently suggested that the substance might play a role in algal control in natural aquatic ecosystems.

Berland *et al.* (1972) examined the toxicity of about 50 strains of bacteria to a variety of marine algae. *Pseudomonas aeruginosa* was found to be particularly inhibitory to *Tetraselmis striata*, a member of the Prasinophyceae. The authors concluded that it was not possible to state that bacteria or their byproducts have important relationships with algae in the oceans.

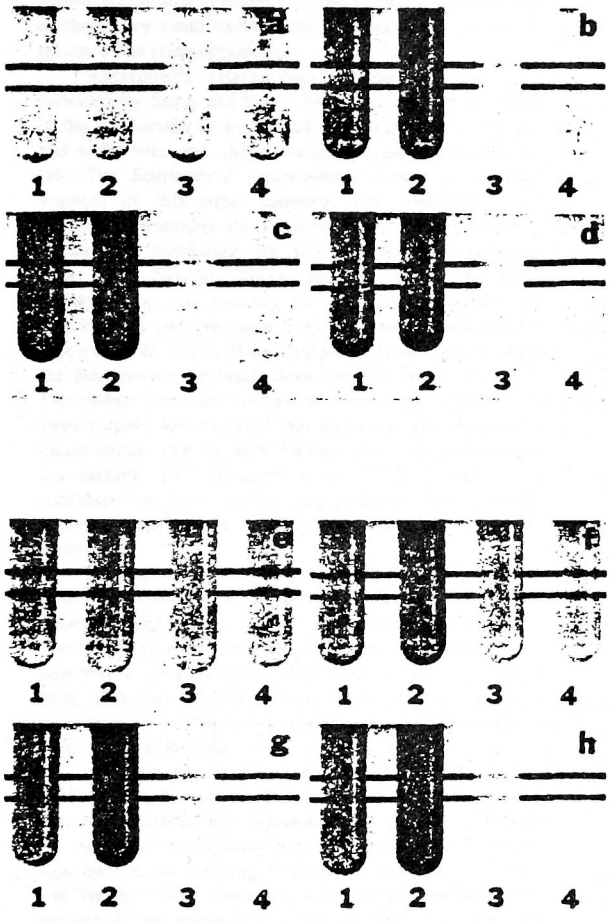
Safferman and Morris (1962) demonstrated that actinomycete filtrates had considerable inhibitory activity against several strains of blue-green algae. This work resulted in the suggestion that these antibiotic substances could be used as algicides. Sladekova and Sladek (1968) supported this idea of using bacterial antibiotic secretions to control algae in the environment.

Similarly, a *Bacillus brevis* strain was shown to produce an extracellular product that caused lysis of both several blue-green algal species and several bacterial species (Reim *et al.* 1974). This non-enzymic substance was quite heat stable, of low molecular weight and was identified as possibly an antibiotic similar to Gramicidin S. Reim *et al.* indicated that the utility of control by an antibiotic system may be questionable due to the difficulty in achieving sufficient concentrations of the inhibitor.

PROPERTIES OF BDELLOVIBRIO BACTERIOVORUS

Recent investigations of a unique bacterium, *Bdellovibrio bacteriovorus*, have demonstrated the effectiveness of this small microorganism in destroying populations of host bacteria (Shilo 1969; Starr and Baigent 1966; Burnham *et al.* 1968; Starr and Seidler, 1971). The occurrence of this organism in the natural environment is widespread and well documented (Shilo 1969).

Since Stolp and Petzold (1962) first discovered the existence of this parasitic bacterium, investigators have been examining microbial parasitism from two approaches: one group has been involved in isolation and characterization of *B. bacteriovorus* from soils and sewage in an attempt to determine the economic importance of this organism in controlling the populations of susceptible bacterial hosts; the other group has been concerned with the basic biochemical, physiological and structural characteristics of this parasitic organism and its hosts to improve our knowledge of its interaction with other bacteria and that of host-parasite relationships in general.



LEGEND

Figure 1. Photographs of (a to d) two day and (e to h) six day old cultures of *p. luridum* mixed with equal volumes of: 1, AB medium; 2, YP medium; 3, 24 hr culture of *B. bacteriovorus* 15143; 4, cell free supernatant from a 24 hr culture of *B. bacteriovorus* 15143. Time sequence: (a,e) 0 hrs, (b,f) 72 hrs, (c,g) 120 hrs, (d,h) 160 hrs.

The name *Bdellovibrio bacteriovorus*, first proposed by Stolp and Starr (Burnham and Robinson 1974; Stolp and Starr 1963) adequately expresses the principal characteristics of the organism: "Bdello" is derived from the Greek word meaning "leech"; "vibrio" denotes its shape; and "bacteriovorus" indicates that it literally eats bacteria. Initially it was reported by Stolp and Starr (1963) that *B. bacteriovorus* was an ectoparasite – that apparently it did not enter the host but obtained its nutrient from a position on the host cell wall. Scherff *et al.* (1966) were the first to show that *B. bacteriovorus* attacks gram negative bacteria by actually penetrating the host bacterium rather than remaining on the outside. These results were supported by Starr and Baigent (1966). Lepin *et al.* (1967) confirmed this endoparasitism by studying a *B. bacteriovorus* attacking *Salmonella typhi* obtained from a polluted river.

Burnham *et al.* (1968), investigating the penetration mechanism in detail, showed that both physical and enzymatic actions combine to cause the localized breakdown of the host cell wall. The evidence to date supports the idea that *B. bacteriovorus* is an endoparasite that eventually penetrates the host cell and multiplies within it. The cycle is completed on release of the new progeny from the host. Further refinement of the life cycle has been reported (Burnham *et al.* 1970).

Until recently, *B. bacteriovorus* was the only bacterium known to parasitize other bacteria; however,

Guelin, *et al.* (1968) isolated a related organism able to attack and penetrate Gram positive bacterial hosts. This is significant because it means that there may be other species of these very small bacteria that are capable of parasitism against other microorganisms.

Antagonistic relationships between bacteria were reviewed by Stolp and Starr (1965), particularly in regard to the production of antimicrobial substances like enzymes and antibiotics and direct microbial attack upon another cell. The *Bdellovibrio bacteriovorus* system is a classic example of this latter category. The mechanisms and enzyme interactions that explain how this parasitism is successfully completed are still not totally understood (Starr and Seidler 1971), but a partial enzymatic understanding has resulted by the recent isolation of muramidases and proteases from *Bdellovibrio* populations (Fackrell *et al.* 1972). These authors point out that by itself the *Bdellovibrio* peptidase does not lyse living cells; only heat killed cells are susceptible to enzyme degradation. They further demonstrated that the site of activity was the mucopeptide layer of both the host, *Spirillum serpens*, and the parasite itself (Fackrell *et al.* 1972). Recently the peptidase has been purified and separated from protease activity completely (J. Robinson, personal communication).

Shilo and Bruff (1965) demonstrated that another species of *Bdellovibrio bacteriovorus* (strain A3.12) was capable of degrading a variety of host bacterial species but only if they had been killed. They postulated that exoenzyme production by bdellovibrios was the second stage of a two stage penetration process, the first being some sort of mechanical damage due to the bdellovibrio's violent attack (Burnham *et al.* 1968).

Bacteriolysis is not solely a characteristic of the bdellovibrios, for a few other groups of bacteria, notably the myxobacteria, the cytophaga, and the actinomycetes are capable of lysing many bacterial strains as well as many algal species. In studying *Myxobacter* strain AL-1, Ensign and Wolfe (1966) described an enzyme possessing both proteolytic and cell-wall lytic activity. These two functions were inseparable upon purification, making the enzyme distinct from that isolated and purified from *Bdellovibrio* (Fackrell *et al.* 1972; J. Robinson, personal communication). Review of actinomycete lysis of other microbes indicates that the responsible enzymes are peptidases (Stolp and Starr 1965; Ghuysen 1968), lending support to the idea that the bdellovibrio factor or aggressin causing the breakdown of the host organism is proteinaceous in nature, and possibly a specific peptidase in activity.

RATIONALE FOR THE CONCEPT OF BDELLOVIBRIO PARASITISM OF ALGAE

Ultrastructural investigations of various species of blue-green algae confirm that this group is procaryotic in structural organization. Thus, they are more closely related to bacteria than they are to other algal forms. The following facts are significant in relating bacterial parasitism of algae with the understanding we now have of the nature of bdellovibrio parasitism: 1) The internal organization of the blue-green algae is procaryotic, similar to that of bacteria (Echlin and Morris 1965; Lang 1968). 2) *B. bacteriovorus* has been shown to parasitize photosynthetic bacteria *(Rhodospirillum rubrum)* (Burger, Drews, and Ladwig, 1968), including *Chromatium* strain D (Burnham unpublished data) so that the presence of photosynthetic thylakoids within the algal cells should not inhibit parasitism. 3) Although the cell wall of blue-green algae has been shown to be structurally distinct from that of typical Gram negative bacteria (Lang 1968), apparently the cell walls of the two types of microorganisms are biochemically very similar (Echlin and Morris 1965). This is particularly important in view of the observations of Varon and Shilo (1969) concerning the possible receptor sites for *Bdellovibrio* attachment. Also significant as far as the receptor sites are concerned are the similarities between bdellovibrio attachment and the attachment of phage to host bacterial cells (Stolp and Starr 1963). In this regard, *B. bacteriovorus* hosts are also susceptible to bacteriophage attack. Furthermore, blue-green algal phage have been isolated which will attach to and eventually lyse species of blue-green algae (Brown 1972; Safferman and Morris 1964, 1967). All of this is evidence for a similar surface biochemistry between bacteria and blue-green algae.

Since it has been shown that both Gram positive and Gram negative bacterial cell walls are penetrated by *B. bacteriovorus* or a related bacterium (Shilo 1969; Starr and Seidler 1971), it is not unreasonable to assume that the slightly different layering of the outer cell wall of most blue-green algal species will also be penetrable by a parasitic species of bacteria.

I believe it helpful to consider this form of biological control as a search for a specific disease—a disease of algae. The present etiologic agent, the bdellovibrio, is highly motile, has a parent/daughter ratio of up to 1/30 and, therefore, can rapidly spread itself through an aquatic environment. This means that *B. bacteriovorus* may be capable of eliminating large populations of algae. This concept of algal control by natural means has been found to be significant in both the laboratory environment and in the natural environment of the algal populations (Safferman and Morris 1964). One of the primary problems of viral control of blue-green algae has been that the phage

is species specific. From my experience with the *B. bacteriovorus* strains ATCC No. 15143 and UKi2, they have much greater cross-reactivity than phage (Burnham *et al.* 1968; Shilo 1969; Starr and Seidler 1971). This fact may be important in determining the type of blue-green algal species found in natural waters.

INTERACTION OF B. BACTERIOVORUS 15143 AND BLUE-GREEN ALGAE

To test the above ideas, *B. bacteriovorus* 15143 was added to a minimally contaminated (bacterially) culture of *Oscillatoria*. The results were startling, for within 30 min. the *Oscillatoria* ceased movement and never regained it during observations which continued until the death of the *Oscillatoria* could be verified by microscopy or respirometry.

My laboratory found that active *Bdellovibrio* cultures as well as culture supernatants (rendered cell free by centrifugation and filtration) are capable of breaking down *Oscillatoria*. The disintegration caused by the enzymatic secretions of the bdellovibrios is extensive, resulting in the loss of all cytoplasmic cell contents and eventual dissolution of the cell wall. In order to eliminate contaminated medium as a cause of this algal lysis, *Oscillatoria* cells were introduced into sterile tubes containing YP (yeast extract and peptone) medium or Nutrient Broth and observed for a period of days. In spite of the fact that these cultures also became overgrown with contaminating bacteria, the condition of the algae remained normal in terms of motility and cellular organization.

The physiological condition of the *Oscillatoria* was observed immediately after adding the bdellovibrios to determine if any immediate damage not visible by microscopy was occurring. The results indicate that both respiration and photosynthesis were within normal values after 1.5 hours exposure to the bdellovibrios. Because it is difficult to be sure of respiration and photosynthesis data when contaminating bacteria are present, cultures of the blue-green alga *Phormidium* were employed as hosts for these experiments with the bdellovibrios. The cultures of *Phormidium* were bacteria-free and allowed accurate measurment of oxygen uptake and evolution.

Four day old cultures of *P. luridum* grown in a mineral salts base were routinely utilized as hosts for the *B. bacteriovorus* 15143. If equal amounts of *P. luridum* culture are added to a 24 hour *B. bacteriovorus* culture, structural alterations in the blue-green algae could be observed continuously until the algae lysed after four days of interaction. These structural alterations included formation of refractile granules, intracellular spaces, intercellular spaces, cell swelling, breakdown of the trichomes, and finally disintegration of the algal cell. During the four day period the turbidity of the mixed cultures gradually decreased. Fig. 1 shows two series of photographs of cultures of *P. luridum*, a–d, representing a low density culture (approximately 10^7 cells/ml), and e–h, a high density culture (approximately 10^9 cells/ml). In all cases, the controls (tubes 1 and 2) showed increasing cell densities throughout the week long experiment. The tubes with viable bdellovibrios (No. 3) showed total inhibition of growth in the low density series, and lysis of the *P. luridum* in the high density series. Similar results can be observed for the cell free culture supernatant of *B. bacteriovorus* 15143. The improved clarity of the tubes showing lysis by factors contained in the cell supernatant is due to the lack of bacteria in the system as compared to Tube 3 containing the bdellovibrios. The interaction showed measurable chlorophyll *a* in the system to decrease markedly and the amount of protease activity in the cultures to increase (Burnham and Stetak 1972; Burnham *et al.* 1975).

Because of the structural and pigment changes, the photosynthetic activity of the *P. luridum* was monitored. Over 90 percent of all 0_2 production was inhibited by the *B. bacteriovorus* whole cells or by a cell-free supernatant prepared from a 24 hour *bdellovibrio* culture. Similar to the results with myxobacteria (Daft and Stewart 1971), placing the *Bdellovibrio* on algal lawns resulted in plaques (Burnham and Stetak 1972; Burnham 1973).

Electron microscopy showed the gradual degradation of the photosynthetic thylakoids with a splitting of the paired photosynthetic membranes and the intermembranous space being filled with a low density homogenous substance. This splitting was shown to continue until the membranes disrupted (Burnham and Stetak 1972; Burnham 1973). The end result was a mass of membrane fragments mixed with ribosomes and other cellular debris.

When the 24 hour *Bdellovibrio* culture supernatant was autoclaved, the photosynthetic inhibitory activity was retained. The autoclaved material inhibited over 90 percent of *P. luridum* and *Microcystic aeruginosa* 0_2 activity in 15 min. Interestingly, cell respiration was not inhibited. The inhibitor was shown to have a pH optimum of 9.5 and did not have any activity below pH 7.0 or above 11.0. Ultramembrane filtration has shown the inhibitor to be less than 10,000 MW. It is very sensitive to membrane dialysis or gel filtration techniques. Approximately 50 percent of the inhibitory activity is lost when the bdellovibrio culture supernatant is subjected to protease activity (Burnham *et al.* 1974).

Recently, comparisons of the heat resistant bdellovibrio photosynthetic inhibitor with dichloromethyl urea (DCMU) and potassium cyanide (KCN) showed a close resemblance to the action of DCMU. Ultra- structure 0_2

evolution and *in vivo* ferricyanide reduction were used to measure the similarities. These results suggested that the bdellovibrio-produced inhibitor could operate by shutting down photosystem II in *P. luridum* (Sun *et al.*1975).

That both bdellovibrios and blue-green algae are common in natural water habitats has been well documented. This, plus evidence that bdellovibrios directly affect *Phormidium* photosynthetic mechanisms and are capable of lysing several species of blue-green algae in an agitated liquid environment, suggests that these bacteria may play a role in the control of blue-green algal populations in their aquatic ecosystems. The natural tendency of *B. bacteriovorus* to interact with other microorganisms (Shilo 1969; Starr and Seidler 1971) makes the relationship indicated here a more likely phenomenon in nature.

ACKNOWLEDGEMENT

Appreciation is expressed to Mr. Greg Locher, Mr. Donald Sun and Mr. Tom Stetak for their expert technical assistance in obtaining the data described within this report. This work has been supported in part by grants from the Toledo Edison Company; Owens-Illinois Corporation; Ohio Water Resources Research, U.S. Department of Interior; and a NIH General Research Support Grant.

REFERENCES

Berland, B.R., D.J. Bonin and S.Y. Maestrini. 1972. Are some bacteria toxic for marine algae? *Marine Biol.*, *12*:189-193.

Brown, R.M. 1972. Algal Viruses. *Adv. Vir. Res.*, *17*:243-277.

Burger, A., G. Drews, and R. Ladwig. 1968. Host range and infection cycle of a newly isolated strain of *Bdellovibrio bacteriovorus*. *Arch. Mikrobiol.*, *61*:261-279.

Burnham, J.C. 1973. Bacterial control of aquatic algae. Project Completion Report No. 414X. State of Ohio Water Resources Center, U.S. Dept. of Interior.

Burnham, J.C., T. Hashimoto, and S.F. Conti. 1968. Electron microscopic observations on the penetration of *Bdellovibrio bacteriovorus* into gram-negative bacterial hosts. *J. Bacteriol.*, *96*:1366-1381.

Burnham, J.C., T. Hashimoto and S.F. Conti. 1970. Ultrastructure and cell division of a facultatively parasitic strain of *Bdellovibrio bacteriovorus*. *J. Bacteriol.*, *101*:997-1004.

Burnham, J.C. and J. Robinson. 1974. The genus and species of *Bdellovibrio*. In *Bergey's manual of determinative bacteriology*, 8th ed. N. Gibbons and R.E. Buchanan, eds. Williams and Wilkins, Baltimore, Md.

Burnham, J.C. and T. Stetak. 1972. Observations on the interaction of *Bdellovibrio bacteriovorus* with two species of blue-green algae. Abs. Ann. Mtg. Am. Soc. Microbiol. p. 33 (G18).

Burnham, J.C., G. Locher, and T. Stetak. 1975. Lysis of *Phormidium luridum* by *Bdellovibrio bacteriovorus*. *J. Phycol.* (Submitted)

Burnham, J.C., D.C. Sun and T. Stetak. 1974. Biological properties of a *Bdellovibrio*-produced inhibitor of blue-green algae. Abs. Ann. Mtg. Am. Soc. Microbiol. G255.

Daft, M.J. and W.D.P. Stewart. 1971. Bacterial pathogens of fresh water blue-green algae. *New Phytol.*, *70*:819-829.

Echlin, P. and L. Morris. 1965. The relationship between blue-green algae and bacteria. *Biol. Rev. Cambridge Phil. Soc.*, *40*:143-187.

Ensign, J.C. and R.S. Wolfe. 1966. Characterization of a small proteolytic enzyme which lyses bacterial cell walls. *J. Bacteriol.*, *91*:524-534.

Fackrell, H.B., G.K. Cambell, J.C.C. Huang, and J. Robinson. 1972. Evidence for a lytic enzyme produced by *Bdellovibrio bacteriovorus* 6-5-S. *Can. J. Microbiol.*, *18*:281-287.

Fenner, F. 1959. Myxomatosis. *Brit. Med. Bull.*, *15*:240-245.

Fogg, G. E., W.D.P. Stewart, P. Fay and A.E. Walsby. 1973. *The blue-green algae*. 297 p. Academic Press, N.Y.

Ghuysen, J.M. 1968. Use of bacteriolytic enzymes in determination of wall structure and their role in cell metabolism. *Bact. Rev.*, *32*:425-464.

Granhall, U. and B. Berg. 1972. Antimicrobial effects of *Cellvibrio* on blue-green algae. *Arch. Mikrobiol.*, *84*:234-242.

Guelin, A., P. Lepine, and D. Lamblin and J. Sisman. 1968. Isolation d'un parasite bacterien actif sur les germes gram-positifs a partir d'echantillons d'eau polluee. *Compt. Rendu., 266*:2508-2509.

Lang, N.J. 1968. The fine structure of the blue-green algae. *Ann. Rev. Microbiol., 22*:15-46.

Lepine, P.M., A. Guelin, J. Sisman, and D. Lamblin. 1967. Etude au microscope electronique de la lyse de *Salmonella* par *Bdellovibrio bacteriovorus*.

Mamkaeva, K.A. 1966. Studies of lysis in cultures of *Chlorella*. *Microbiology,* (USSR; English Transl.), *35*:724-728.

Mitchell, R. 1972. *Water pollution microbiology.* Wiley-Interscience, New York.

Reim, R.L., M.S. Shane, and R.E. Cannon. 1974. The characterization of a *Bacillus* capable of blue-green bactericidal activity. *Can. J. Microbiol., 20*:981-986.

Rivers, C. 1964. Virus pesticides. *Discover, 25*:26-31.

Safferman, R.S. and M.E. Morris. 1962. Evolution of natural products for algicidal properties. *Appl. Microbiol., 10*:289-292.

Safferman, R.S. and M.E. Morris. 1964. Control of algae with virus. *J. Am. Water Works Assoc., 56*:127-1224.

Safferman, R.S. and M.E. Morris. 1967. Observations on the occurrence, distribution, and seasonal incidence of blue-green algal viruses. *Appl. Microbiol., 15*:1919-1922.

Scherff, R.H., J.E. DeVay and T.W. Carroll. 1966. Ultrastructure of host-parasite relationships involving reproduction of *Bdellovibrio bacteriovorus* in host bacteria. *Phytopathol., 56*:627-632.

Shea, K. 1971. Infectious cure. *Environment, 13*:43-45.

Shilo, M. 1969. Morphological and physiological aspects of the interaction of *Bdellovibrio* with host bacteria. *Curr. Top. Microbiol. Immunol., 50*:174-204.

Shilo, M. 1970. Lysis of blue-green algae by *Myxobacter. J. Bacteriol., 104*:453-461.

Shilo, M. and B. Bruff. 1965. Lysis of gram-negative bacteria by host independent ectoparasitic *Bdellovibrio bacteriovorus* isolates. *J. Gen. Microbiol., 40*:317-328.

Sladeckova, A. and V. Sladeck. 1968. Algicides–friends or foes? In *Algae, man and the environment* (D. Jackson, ed.) p. 441-458. Syracuse Univ. Press, New York.

Starr, M.P. and N.L. Baigent. 1966. Parasitic interaction of *Bdellovibrio bacteriovorus* with other bacteria. *J. Bacteriol., 91*:2006-2017.

Starr, M.P. and R.J. Seidler. 1971. The bdellovibros. *Ann. Rev. Microbiol., 25*:649-678.

Stewart, J.R. and R.M. Brown. 1969. *Cytophaga* that kills or lyses algae. *Science, 164*:1512-1524.

Stolp, H. and H. Petzold. 1962. Untersuchungen über einen obligat parasitischen Mikroorganismus mit lytischer Aktivität für *Pseudomonas*-Bakterien. *Phytopathol. Z., 45*:364-390.

Stolp, H. and M.P. Starr. 1963. *Bdellovibrio bacteriovorus* gen. et sp. n., a predatory, ectoparasitic, and bacteriolytic microorganism. Ant. van Leeuwenhoek. *J. Microbiol. Serol., 29*:217-248.

Stolp, H. and M.P. Starr. 1965. Bacteriolysis. *Ann. Rev. Microbiol., 19* :79-104.

Sun, D.C., G. Locher and J.C. Burnham. 1975. Comparison of photosynthetic inhibition in spheroplasts and whole cells of *Phormidium luridum*. Abs. Ann. Mtg. Am. Soc. Microbiol.

Varon, M. And M. Shilo. 1969. Interaction of *Bdellovibrio bacteriovorus* and host bacteria. 11. Intracellular growth and development of *Bdellovibrio bacteriovorus* in liquid cultures. *J. Bacteriol., 99*:136-141.

A CYTOCHEMICAL STUDY OF PHOTOSYNTHETIC AND RESPIRATORY ELECTRON REACTIONS IN *PHORMIDIUM LURIDUM* var. *OLIVACAE*

Donald Sun and Jeffrey C. Burnham
Department of Microbiology
Medical College of Ohio
Toledo, Ohio 43699

INTRODUCTION

Recent studies (1, 2, 4, 6, 8, 15, 25) show that cytochemical methods can serve as a rather reliable indicator in monitoring the electron transport activities in cyanobacteria. At the present time, cytochemical methods also represent the best way to determine sites of enzymatic activity in or on membranes under *in situ* or newr *in situ* conditions. This paper examines the dual functional nature of the photosynthetic lamellae of *Phormidium luridum*, as well as the inhibitory toxin produced by *Bdellovibrio bacteriovorus* on the electron transport activities of the photosynthetic lamellae.

In addition to potassium ferricyanide, other cytochemical localization agents used in this study were potassium tellurite, tetranitroblue tetrazolium (TNBT) and 3, 3-diaminobenzidine tetrahydrochloride (DAB). Similar in action to potassium ferricyanide, potassium tellurite and TNBT could act as artificial electron acceptors. In contrast, DAB donates, rather than accepts, electrons from the electron transport systems (22). DAB is a good electron donor in the mitochondria cytochrome system when used to demonstrate the location of cytochrome oxidase and cytochrome c (22). It can also act as an electron donor in some parts of the electron transport chain in photosynthesis (20). As electrons are donated, DAB is oxidized to a water insoluble, lipid insoluble osmiophilic polymer which reliably localizes the sites of activity (22).

These localization agents were coupled with a variety of cyanobacterial inhibitors in order to furnish data about the *in situ* locations which were affected by a particular inhibitor. This research will provide comparative data on the inhibition of cyanobacteria by known chemical inhibitory agents and the inhibitory toxins produced by *Bdellovibrio bacteriovorus* (3). Through this study, further understanding of the electron reduction activities and

their relationship with the ultrastructural organization of cyanobacteria can also be achieved.

This should help in the evaluation of this bacterium as a possible agent for the control of aqueous cyanobacterial populations.

MATERIALS AND METHODS

The growth and maintenance of Phormidium luridum, var. olivacae Borech, and Bdellovibrio bacteriovorus (strain No. 15143) have been described (3). The conditions for production of the bdellovibrio toxin in 4x yeast extract (12 g/l)-peptone (2.4 g/l) medium have been described (3), as have the methods used in measuring both cellular photosynthesis and respiration.

Cytochemical Localization: (1) Potassium tellurite. Potassium tellurite was added to late log phase unwashed cells to give a final concentration of 0.1%. Concentration of tellurite higher than 0.1% was found to exhibit inhibitory effects on cyanobacterial growth. Additional cell samples were supplied 0.1% potassium tellurite, 0.4% sodium succinate and 0.01M sodium citrate. All samples were examined after incubation up to 72 h in the light and in the dark according to the method described by Bisalputra et al. (2).

(2) 3, 3-diamnobenzidine tetrahydrochloride (DAB). Late log phase Phormidium luridum cells grown in algae broth were spun down and washed once with 0.05 M sodium potassium phosphate buffer (pH 7.0), and resuspended in the phosphate buffer to their original volume. Five ml of this cell solution were pipetted into corex 30 ml test tubes and the different inhibitors were added.

After incubation in a lighted incubator (30°C) for 1 h, tubes of 5 ml Phormidium phosphate buffer suspension, with or without inhibitors, were placed in a water shaker bath (35°C). Sufficient amounts of freshly prepared DAB solution (dissolved in 0.05 M phosphate buffer, pH 7.0) were added to give a final concentration of 0.025% (9); the reaction was initiated by exposing the cell mixtures to the tungsten light source. The cells turned from green to a brownish-green color after 10 minutes of reaction due to the oxidation of DAB by cytochrome oxidase. The algal cells were centrifuged, placed in agar blocks, fixed with 2% O_5O_4 in RK buffer (pH 6.6), dehydrated in acetone and

embedded in Epon.

Control experiments included: (a) no inhibitor added to the Phormidium phosphate suspension; (b) omission of diaminobenzidine tetrahydrochloride in the cell suspension; and (c) incubation and reaction of cells with DAB in the dark (without inhibitor).

(3) Tetranitroblue tetrazolium (TNBT). Because TNBT is relatively insoluble in water, it was put into solution by the method of Sedar and Burke (21).

Late log phase Phormidium were harvested and washed with 0.2 N sodium potassium phosphate buffer (pH 7.2), centrifuged and resuspended in 0.2 N sodium potassium buffer. Cell suspensions were pipetted into corex test tubes (5 ml each) and different inhibitors were added. These tubes were incubated for 1 h in a shaker water bath (30°C). Localization reactions were initiated by adding enough TNBT solution to each tube to constitute a concentration of 0.4 mg/ml and exposing the cells to the light source. After 15 minutes, reactions were terminated by centrifugation of cells at 4°C in the dark. The supernatant was discarded and the cells were placed in agar blocks, fixed with 2% O_5O_4 in RK (pH 6.6) buffer for 12 to 16 h, dehydrated with acetone or cold ethanol and embedded in Epon.

The procedure for dark reactions was the same. In addition, 0.2 N disodium succinate was added to all experimental tubes. The duration of incubation with or without inhibitor was 1 h and the cytochemical localization reaction was carried out for 30 minutes instead of 15 minutes.

The control experiments, run both in the light and in the dark, were cells in sodium potassium phosphate or in phosphate buffer with TNBT without disodium succinate.

(4) Inhibitors: In addition to dichlorophenyl methylurea (DCMU), which inhibits electron transport from cytochrome b to plastoquinone and cytochrome c (8), potassium cyanide (KCN), which inhibits electron transport in the cytochrome system (4), and Bdellovibrio bacteriovorus toxin (BdAS), which inhibits photosynthetic activity, stimulates photorespiration, and only partially inhibits dark respiratory activity (3), other algal inhibitors used were rotenone,which inhibits NADPH oxidation (17), ouabain, which affects potassium influx and sodium efflux from cell membranes (17, 19) and the sodium-potassium activated ATPase (19); O-phenanthroline, which inhibits complete photosynthesis (14); polymyxin B, which interacts with 'target sites' (phospholipid) in the membrane and yields irreversible breakdown of the permeability barrier of cell membrane (13); amphotericin B, which inhibits photosynthetic electron transfer by releasing plastocyanin from its site in the membrane (13a). Cultures with different inhibitors and controls were tested for their photosynthetic and respiratory activities, i.e., O_2 production and O_2 uptake, reduction of ferricyanide to ferrocyanide (for monitoring of electron transport systems) and cytochemical localization reactions.

(5) Chemicals. The following chemicals were obtained from Sigma, St. Louis, Missouri: rotenone, ouabain. Diaminobenzidine tetrahydrochloride (DAB) and tetranitroblue tetrazolium chloride (TNBT) were obtained from Polysciences, Inc., Warrington, Pennsylvania. Amphotericin B was obtained from E. R. Squibb and Sons, Inc. Polymyxin B sulfate was obtained from Burroughs Wellcome Company. 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea (DCMU) was obtained from the DuPont Chemical Company.

RESULTS

(1) Photosynthesis and respiration. O-phenanthroline, DCMU, KCN, DCMU+KCN and BdAS caused severe inhibition of photosynthetic and respiratory activities of P. luridum. In all cases, photosynthetic activity was more sensitive to these inhibitors than was cellular respiration. Photosynthetic oxygen production by O-phenanthroline-treated cells was less than 10% compared to that of the control. DCMU, KCN and DCMU+KCN-treated cells did not produce oxygen under lighted conditions. BdAS caused photorespiration, i.e., oxygen consumption was actually faster under light conditions than under dark conditions.

After 1 h incubation, rotenone, ouabain and amphotericin B did not seem to affect either the photosynthetic or respiratory activity of P. luridum. In fact, oxygen production was slightly enhanced compared to the control cells. Incubation of P. luridum with rotenone for 24 h showed some inhibition of photosynthetic activity (Table 1).

(2) Ferricyanide reduction. The average value of ferricyanide reduction by whole cells was 2.13 uM/mg Chl a/min (23). Whole cells treated with DCMU (5 uM), KCN (25 uM), ouabain (1 uM) and rotenone (10^{-4}M) caused different degrees of inhibition of ferrycyanide reduction. However, cells treated with amphotericin B (10 ug/ml or 200 mg/ml), O-phenanthroline and BdAS did not inhibit reduction of ferricyanide; instead, increased amounts of ferricyanide were being reduced (Table 2). Cells treated with polymyxin B also resulted in an increase of ferricyanide activity (23).

(3) Cytochemical localization. Figure 1 shows a P. luridum cell which was exposed to the reaction mixture under cytochemical reaction conditions, but without the localization agent or any inhibitors. The cell indicates that this treatment had very little adverse effect on the ultrastructure of

the cell. Noticeable changes indicated bleb formation on the cell surface and a darkening of the intrathylakoidal region. This was possibly due to the concentration of ferricyanide which was present in the reaction mixture (but no copper sulfate) in this region.

(a) Potassium tellurite. Zero-hour and 4 h samples did not show any deposition of reduced potassium tellurite. At 12 and 24 h, light-grown cells became visibly darker due to tellurite reduction. Samples taken at 24 h showed deposition of reduced potassium tellurite on or adjacent to the photosynthetic membrane (Fig 2). Although this could also be seen in cultures incubated in the dark, deposition of reduced potassium tellurite seemed faster and more abundant under lighted conditions. The quantity and size of tellurite depositions increased with time. No tellurite depositions could be seen in the centroplasm, or on the cytoplasmic granules; however, some deposits were located very close to the cytoplasmic membrane. The dark rod-like precipitates were of various lengths and were found up to 240 nm long. The structure of the rods could be resolved into several dark and light alternating layers, with the dark layers always on the exterior. Their total width varied from 40 nm to 60 nm. No precipitate could be seen in control cells represented by Figure 3.

(b) 3, 3-diaminobenzidine tetrahydrochloride (DAB). Exposure of P. luridum to DAB caused a visible darkening (under lighted conditions) of the cells. The period of incubation with DAB (15 minutes) was chosen to ensure sufficient deposition. Intense staining was observed exclusively in the space between the two closely stacked photosynthetic lamellae (intrathylakoidal retion). The depositions were linear, and were not droplet in character. Staining of intrathylakoidal region extended throughout the entire thylakoidal system (Fig 4). Figure 4 illustrates that although the staining

was variable in intensity, it was in most instances not interrupted. Figure 4 also shows that the periplasm and centroplasm were free from any deposition.

DCMU did not appear to inhibit the photooxidation of DAB (Fig 5). Similarly, BdAS also did not inhibit oxidation of DAB. When the reaction mixture with BdAS was adjusted to pH 9.0, a slight degree of inhibition of DAB oxidation was exhibited, i.e., the continuity of DAB deposition was interrupted (Fig 6). A control reaction with a pH of 9.0 without BdAS did not affect DAB deposition. KCN (25 mM) almost completely inhibited the staining of the lamellae system (Fig 7), a good indication that oxidation of DAB was very sensitive to the presence of KCN.

Incubation of P. luridum without inhibitors in the dark with DAB also showed some deposition of the oxidized products in the intrathylakoidal spaces.

(c) Tetranitroblue tetrazolium (TNBT). Under lighted conditions, P. luridum incubated for 15 minutes in the presence of TNBT in 0.2 N sodium potassium phosphate, pH 7.2, evidenced visible TNBT reduction within 30 seconds. The green cell suspension became a constant brownish-green after approximately 5 minutes. In tubes where inhibitor was added to the incubation medium, there usually was an obvious change in the visible reduction of TNBT. No change occurred in the color of cells incubated with polymyxin B plus amphotericin B.

Electron micrographs of P. luridum cells treated with TNBT showed a darkening of the outer photosynthetic membrane which faced the cytoplasm (Fig 8, arrow). Staining of the outer photosynthetic membrane in most instances was not continuous. Only about 20% to 40% of the lamellar membrane which faced the cytoplasm were stained with TNBT. No staining by TNBT was apparent in the intrathylakoidal region. Electron opaque particles of various sizes (approximately 3.5 nm to 32 nm) can be seen attached to the photosyn-

thetic lamellae as located at the proximity of the membrane system. Quite often these particles were seen in direct contact with the stained outer membrane of the lamellae (Fig 8). Furthermore, in micrographs of P. luridum in reaction mixture without TNBT (controls), no such particles could be found. The surface of the inner cytoplasmic membrane was usually stained with reduced TNBT (Fig 7). The electron density and uniformity of stain on the inner surface of cytoplasmic membrane was comparatively better than on the outer surface of photosynthetic lamellae. Electron-dense particles were often seen in the LIV layer of the cell wall (Fig 9).

P. luridum incubated under light conditions in reaction mixture plus DCMU (5 uM) for one hour prior to addition of TNBT in most cases did not appear to affect reduction of TNBT.

Cells incubated with DCN (25 uM) (not shown) or DCMU (5 uM) plus KCN (Fig 10) showed relatively heavy deposition of electron-dense lamellae. The amount of outer membrane of the photosynthetic lamellae and inner membrane of the plasmolemma stained with reduced TNBT were comparatively less than in control cells.

Cells treated with rotenone (10^{-4}M) and O-phenanthroline (1 mM) under lighted conditions showed staining of the outer membrane of the lamellar system and inner surface of the plasmolemma. Relatively large quantities of particulate deposition attached to or located close to the lamellae were also conspicuous (Fig 11). Deposition on these treated cells (with rotenone or O-phenanthroline) was only slightly less than in cells without treatment of any inhibitor. Cells treated with rotenone in the dark showed substantial ininhibion of TNBT reduction; O-phenanthroline-treated cells in the dark also showed a slight decrease of deposition of the reduced product.

Reduction of TNBT was severely inhibited in cells treated with ouabain (1mM) or polymyxin B (10 ug/ml). Most cells did not show staining of the membrane systems nor deposition of electron-opaque particulates in the proximity of the photosynthetic lamellae. A fair number of P. luridum were lysed when treated with polymyxin B. Cells treated with BdAS appeared to be similar to those treated with rotenone and O-phenanthroline, but the overall impression was that the inhibitory effect of BdAS was slightly more than that of rotenone, etc. A tendency for intrathylakoidal dilution with eventual formation of intrathylakoidal vesicles was present in cells treated with BdAS as has been previously reported.

Cells treated with polymyxin B (10 ug/ml) plus amphotericin B (10 ug/ml) were lysed soon after the addition of TNBT. No intact cells could be found in the sample prepared for electron microscopy. The cell wall and cell membrane were completely disintegrated and only remnants of photosynthetic membrane remained, which were arranged in somewhat broken concentric rings completely void of cytoplasmic materials. The only constituents remaining within these membranous skeletons were hexagonal-shaped and spherical-shaped granules. Deposition of darker particles were seen in the core region of the spherical granules. The nature and origin of these depositions were not clear.

P. luridum incubated in the dark for 30 min in the presence of TNBT (0.4 mg/0.1 ml), 0.2 N sodium potassium phosphate, pH 7.2, and sodium succinate (0.2 N) also resulted in reduction of TNBT. Cells treated with TNBT without inhibitor appeared to be identical under light or dark conditions. Cytochemical localization in cells treated with DCMU, KCN or DCMU plus KCN prior to the addition of TNBT did not inhibit TNBT reduction. Deposition of reduced TNBT could easily be seen.

Incubation of cells with rotenone (10^{-4}M), ouabain (1 mM) and O-phenanthroline in the dark showed a substantial amount of inhibition of TNBT reduction.

Polymyxin B did not appreciably inhibit reduction of TNBT in the dark. Most of the polymyxin B-treated cells showed fair amounts of reduced TNBT deposition.

Polymyxin B plus amphotericin B-treated cells reacted the same in the dark as in the light.

Cells treated with BdAS in the dark only showed a minor interference of TNBT reduction. Staining of cytoplasmic membrane and photosynthetic lamellae which faced the cytoplasm was quite evident. Deposition of electron-dense particulates on or close to photosynthetic lamellae could also be seen. Considerable amounts of electron-dense deposition was present in the region (LIV) between the mucopeptide and LIV layer.

DISCUSSION

Addition of inhibitors such as DCMU, KCN, O-phenanthroline and DCMU plus KCN to P. luridum cells greatly reduced photosynthetic activity, and to a certain extent, respiratory activity which is consistent with previous data for other cyanobacteria (4, 15). However, rotenone, ouabain and amphotericin B stimulated photosynthetic activity. Autoclaved bdellovibrio supernatant was the only single inhibitor to cause photorespiration under lighted conditions. In the case of DCMU, KCN, ouabain, amphotericin B, and BdAS, reduction of ferricyanide was correlated with the metabolic activity of the cells, e.g., a lower photosynthetic rate corresponded to a lower rate of ferricyanide reduction and vice versa. In the case of O-phenanthroline and rotenone, the metabolic activities of cells were inversely proportional to their ferricyanide reduction rate (Tables 1 and 2). For example, O-phenanthroline did not inhibit photosynthesis and respiration; however, the ferricyanide reduction rate was very slow, whereas rotenone inhibited cellular metabolic activity, but also allowed a high ferricyanide reduction rate.

These results with O-phenanthroline and rotenone could be explained if either inhibitor altered the permeability of the cell. It has been suggested that rotenone could form an intermediate product within the cell which would be able to reduce ferricyanide.

Potassium tellurite has been shown to accept electrons from the respiratory enzyme-succinate dehydrogenase (2). The reduced product was an insoluble and highly electron scattering compound which was deposited only over the reactive sites (2). Hence, potassium tellurite was thought to be an ideal compound for cytochemical localization of electron transport activities. Bisalputra et al. (2) used this chemical to locate photosynthetic and respiratory electron transport in Nostoc sphaericum. Experiments with P. luridum

showing that potassium tellurite was reduced in both light and dark conditions demonstrated that photosynthetic lamellae in those cyanobacteria, as in Nostoc sphaericum (2), were the sites of respiration. Potassium tellurite depositions on the lamellae could also be seen in dark incubated cells, both with and without added sodium succinate. It is believed that this reduction in the absence of added succinate was due to the presence of endogenous succinate (2) within the cells.

Distribution of the reaction product of DAB occurred linearly along the P. luridum photosynthetic thylakoids. Use of DAB made possible the demonstration of peroxidases (5), cytochrome c oxidase (22), and photooxidation by chloroplasts (20). Other oxidases do not react with DAB (22).

In the light, oxidation of DAB was due to photooxidation by photosynthetic lamellae and/or peroxidase. In the absence of light, oxidation of DAB was due to cytochrome c oxidase (22). In P. luridum, electron-dense products in the light and in the dark were located exclusively in the intrathylakoidal region, suggesting that cytochrome c and its oxidase were located on the inner surface of the photosynthetic thylakoidal membrane.

Seligman, et al. (22) demonstrated cytochemically the cytochrome oxidase activity with DAB and concluded that oxidation of DAB was on the outer surface of the inner mitochondrial membrane; in other words, depositions of oxidized DAB was within the intracristal spaces of mitochondria in eukaryotic cells. As far as respiration-associated electron transport was concerned, the similarity of function of the intracristal spaces of mitochondria in eukaryotic cells and the intrathylakoidal spaces in cyanobacteria are reinforced in these experiments.

KCN (25 uM) almost completely prevented the oxidation of DAB. This suggests that metalloenzymes are involved in the reaction and KCN may act as

an antioxidant (20). It could also serve as an indication that staining with DAB was due to cytochrome oxidase activity which was sensitive to KCN (22).

BdAS also prevented oxidation of DAB, but to a much lesser extent than KCN. This would suggest that BdAS could be acting as a mild antioxidant in this situation.

The TNBT compounds accept electrons directly from the respiratory chain, possibly at a step before coenzyme Q and cytochrome c (16, 21). The use of TNBT to localize the succinic dehydrogenase in bacteria and cyanobacteria (2, 16) is well documented.

Seligman et al. (22) reported localization of succinic dehydrogenase (SDH) activity with tetrazolium salt. Succinic dehydrogenase-cytochrome b activity appears to be located on the same surface as the inner mitochondrial membrane as noted for cytochrome c activity with DAB. However, reduced TNBT deposits were not seen in the intrathylakoidal spaces of photosynthetic lamellae in P. luridum. Instead, an aggregate of deposits could be seen on the thylakoidal membranes facing the cytoplasm. Opacity of the lamellar structure appeared higher than normal, owing to the deposition of diformazan, in agreement with Leene's observation of TNBT reduction in Bacillus subtilus (16). Electron-dense particles in the interthylakoidal spaces of the P. luridum lamellar system show a location remarkably similar to that reported for phycobilisomes in many cyanobacteria (26).

Inhibition of the TNBT reduction by rotenone-treated cells in the dark was consistent with the fact that rotenone inhibits $NADPH_2$ oxidation (17).

Ouabain did not inhibit photosynthetic or respiratory activities of P. luridum; however, it completely inhibited TNBT reduction. Inhibition of TNBT reduction by ouabain possibly did not represent inhibition of the electron transport system. Ouabain is known to inhibit the activities of the Na/K

exchange pump and the sodium-potassium activated ATPase (18). This could have altered cell membrane properties and resulted in the establishment of intracellular polarities (18). Failure of ouabain-treated cells to exhibit reduced TNBT localization is possibly due to such disturbed intracellular polarities.

The different cytochemical localization agents used in P. luridum showed consistent results in that the localization sites appeared in the same position for the agent measuring the same reaction. Differing degrees of sensitivity and specificity were obtained. For example, potassium tellurite, indicating succinic dehydrogenase activity, formed a heavy precipitate covering both paired photosynthetic membranes and the intrathylakoidal space between; whereas copper ferrocyanide (23), localizing the same enzyme in P. luridum, showed the enzyme to be directly on the cytoplasmic surface of the thylakoids with some reaction occurring in the intrathylakoidal space.

The other cytochemical agent used with P. luridum was DAB, which indicated the location of cytochrome c and its oxidase. In cyanobacteria, cytochrome c is a participant in both photosynthetic and respiratory electron transport, which would be consistent with DAB depositions in both the light and the dark. Diaminobenzidine depositions are clearly within the intrathylakoidal space and not in any other cellular site.

In chloroplasts, DAB deposition was seen mostly continuous on the grama and stroma membrane system. This DAB deposition in chloroplasts indicated the localization of either a photooxidizing process, peroxidase, or cytochrome (in place of cytochrome c) (6). Copper ferrocyanide deposition was also seen on both the grama and stroma lamellae (6). In most instances, this deposition was seen as amorphous patches filling the interthylakoidal space.

In our studies, cytochemical localization with copper ferrocyanide and DAB in the dark by P. luridum indicates an analogy of the intracristal space

in mitochondria and the intrathylakoidal space in cyanobacteria. This observation substantiates the finding of Bisalputra et al. (2) that photosynthetic lamellae in cyanobacteria are also the sites for respiratory electron transport. It also introduces the possibility of functional segregation of lamellar systems which can be identified by cytochemical techniques.

The analogous cytochrome in the cyanobacteria respiratory electron transport to eukaryotic cytochrome b would be cytochrome o, and in cyanobacterial photosynthesis, would be cytochrome b. Localization of reduced TNBT occurred in both light and dark (even though dark reactions were much slower with less precipitate) could be explained if TNBT reacts with cytochrome b in the light and with cytochrome o in the dark.

Haydon (10) and Seligman et al. (22) also compared the results from DAB with the localization of succinic dehydrogenase-cytochrome b activity located on the same surface of the inner mitochondria membrane as noted for cytochrome c activity.

In P. luridum, however, TNBT depositions were seen on the surface of the photosynthetic membrane facing the cytoplasm, inner surface of cytoplasmic membrane, in the cell wall layers LII (mucopeptide) and LIV (lipopllysaccharide), and in the cytoplasm between photosynthetic lamellae, suggesting a different distribution pattern for succinic dehydrogenase in the cyanobacteria from that found in mitochondria. It is also apparent from the precipitate locations that the succinic dehydrogenase in P. luridum is not as closely bound to the cytoplasmic-photosynthetic membrane structures as it is in the bacteria.

The sharing of electron transport components and pathways between photosynthesis and respiration has been postulated (1, 15). The data presented for P. luridum would support this concept.

One of the major problems that our present methodology does not permit is

a direct cytochemical measurement of photosynthetic transport with the total blockage of respiration. As described in our previous paper (23), copper ferricyanide does the reverse in the presence of light, demonstrating respiratory electron transport with the blockage of photosynthesis. Until a unique cytochemical agent is designed, the definite cytochemistry of photosynthesis will be impossible.

Most of the effects of the chemical inhibitors used in this investigation are known (see Materials and Methods). However, the inhibitory effects of BdAS which so profoundly affect physiological activities of the cyanobacteria is still an unknown process. Autoclaved bdellovibrio toxin caused the P. luridum , under lighted conditions, to undergo photorespiration, instead of photosynthesis (3). Comparing the results of BdAS treatment with that of DCMU, KCN, ouabain, rotenone and polymyxin B, the bdellovibrio toxin has to be regarded as a unique inhibitor.

Developing the bdellovibrio toxin or a similar bacterial algal toxin is considered a long-term goal for this research on cyanobacterial control. Without a doubt, environmental control through microbiological means would be a most desirable alternative to the present technology employing chemical inhibitors.

TABLE 1

Effect of inhibitors on the photosynthesis and respiration of P. luridum whole cells under reaction conditions for electron cytochemistry.

Inhibitor	Lighted conditions ul $O_2 \cdot mg\ Chla^{-1} \cdot min^{-1}$	%	Dark conditions ul $O_2 \cdot mg\ Chla^{-1} \cdot min^{-1}$	% %
None (control in AB)	7.0	100	-1.4	100
None (control in rx misture	5.4	77	-1.9	136
O-phenanthroline*	0.4	6	-0.6	43
rotenone*	7.1	101	-1.3	93
ouabain*	7.7	110	-1.2	86
amphotericin B*	7.5	107	-1.1	79
DCMU*	-0.2	- 3	-0.7	50
KCN*	-0.1	- 1	-0.9	64
DCMU+KCN*	0.0	0	-1.5	
BdAS*	-1.8	- 26	-0.7	50

*suspended in cytochemical reaction mixture described in Materials and Methods

TABLE 2

Rate of ferricyanide reduction by *P. luridum* whole cells and effects of inhibitors in cytochemical reaction conditions.

Inhibitor	Lighted conditions $uM \cdot mg\ Chla^{-1} \cdot min^{-1}$	%
None (control in rx mix)	2.1	100
Amphotericin B (10 ug/ml)	5.5	262
Amphotericin B (20 ug/ml)	10.4	495
Rotenone (.1 mM)	0.3	14
Ouabain (1.0 mM)	1.8	86
O-phenanthroline (1.0 mMO	3.2	152
KCN (50 mM)*	1.3	62
DCMU (5 uM)*	1.7	81
BdAS	3.1	148

*From Sun *et al*.

ACKNOWLEDGEMENTS

Funds for the support of this study were provided by a Title I Grant from the Ohio Water Resources Center, Office of Water Research and Technology, U. S. Department of the Interior and the Toledo Edison Corporation, Toledo, Ohio.

The authors wish to thank Mr. Gregory Locher and Mrs. Barbara Highison for their technical assistance.

LEGENDS FOR FIGURES

PLATE I

Fig 1. A cross-section of *P. luridum* showing that after 24 h incubation in the cytochemical reaction mixture, continuing ferricyanide, but neither the localization agent (copper sulfate) nor any inhibitor, the cell retains its cytoplasmic integrity. The arrow indicates the slight increase in density of the intrathylakoidal space which appears after this extensive treatment. Note also the blebs of lipopolysaccharide which appear on the surface of the cell wall. All bars represent 1 um unless otherwise noted.

Fig 2. *P. luridum* showing the results of a 24 hr section for reduction of 0.1% potassium tellurite. Arrow indicates tellurite crystals to be located primarily in the intrathylakoidal region.

Fig 3. This *P. luridum* cell represents a control cell run parallel to the cell in Fig 2, but without the tellurite. Note the absence of any electron-dense crystals.

Fig 4. Higher magnification of a portion of a *P. luridum* cell showing deposition of DAB in the intrathylakoidal region.

Fig 5. *P. luridum* cell after incubation for 15 min in a cytochemical reaction mixture containing DAB and 5 mM DCMU.

Fig 6. *P. luridum* cell after incubation for 15 min in cytochemical reaction mixture containing DAB and BdAS. Note the characteristic interruptions (arrows) produced by this inhibitor.

PLATE II

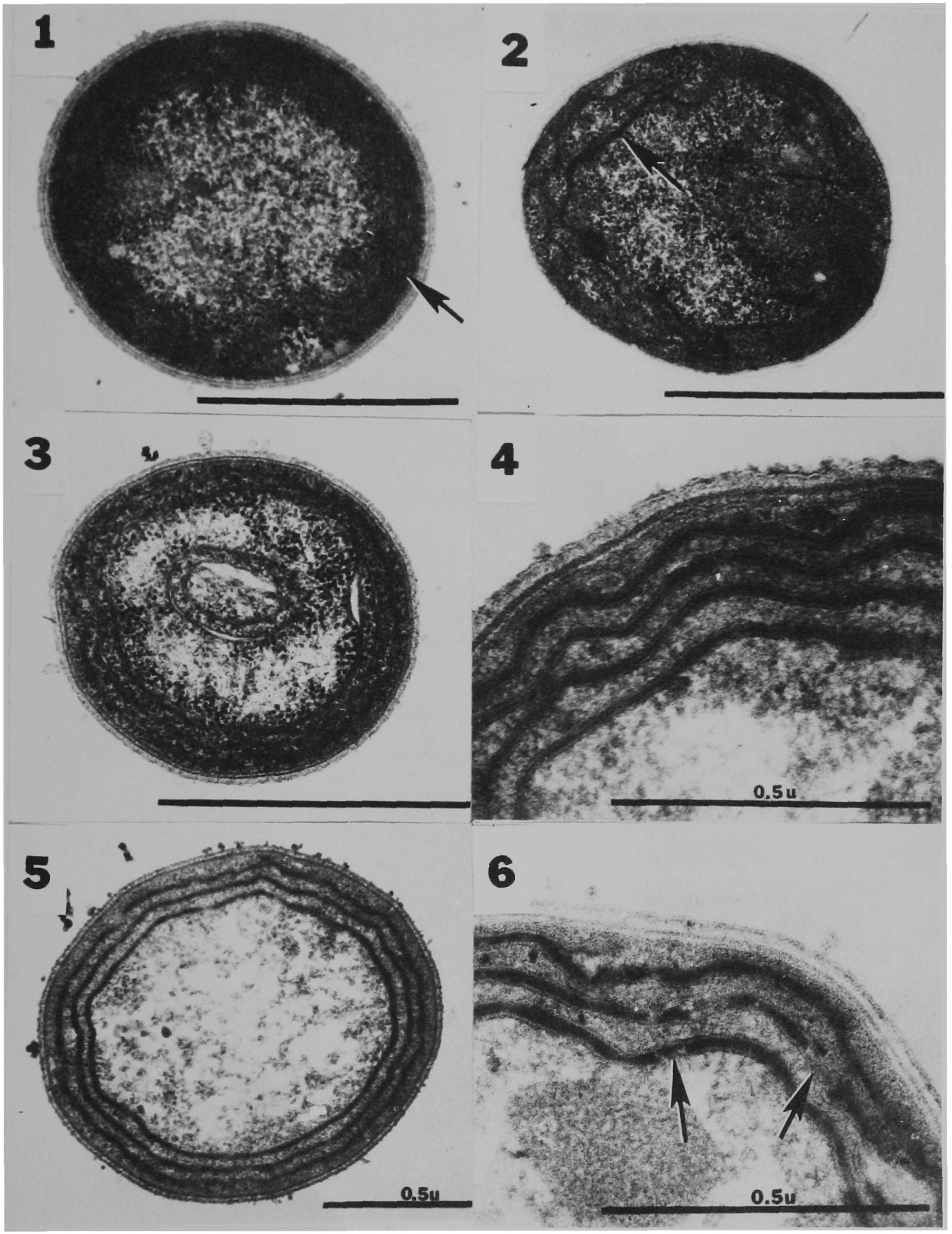
Fig 7. Higher magnification of a P. luridum cell after 15 min lighted incubation in a cytochemical reaction with DAB and 25 mM KCN. Note absence of dense precipitate.

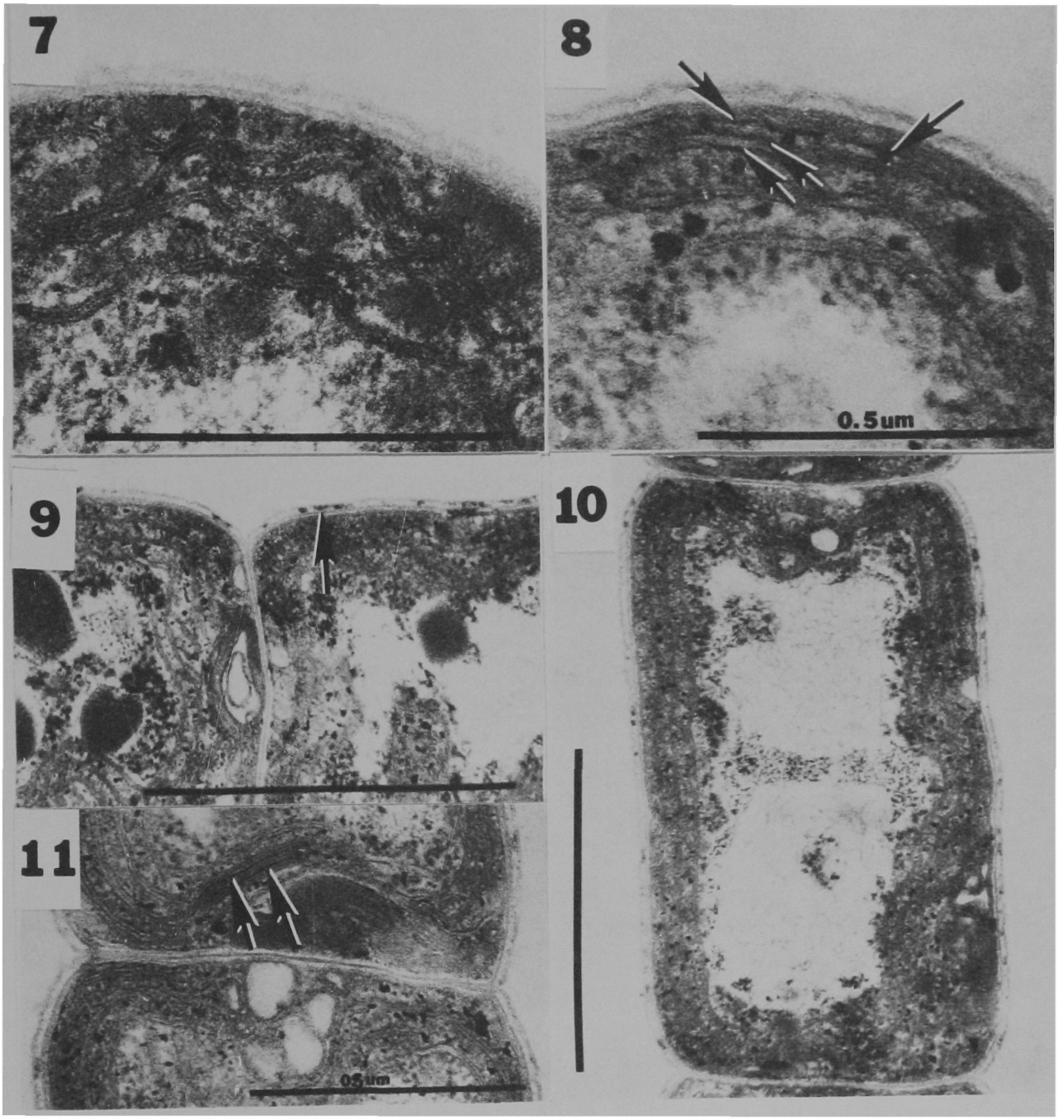
Fig 8. Higher magnification of P. luridum after 15 min lighted incubation with TNBT. Note that the surface of the thylakoidal membrane that faces the cytoplasm is stained with reduced TNBT precipitate (arrows). Electron-dense particles were also frequently seen in close or direct contact with the stained membrane surface.

Fig 9. This micrograph shows that P. luridum contains reduced TNBT deposits in the cell wall between the lipopolysaccharide and mucopeptide layers (arrow).

Fig 10. P. luridum treated with KCN (25 mM) and DCMU (5 uM) during lighted cytochemical reaction with TNBT. No inhibition of TNBT reduction is noticable. Aggregation of electron-dense droplet material can be seen in the centroplasm. This material appeared to be smaller compared to the larger TNBT particles in the vicinity of the thylakoidal membranes.

Fig 11. O-phenanthroline (1 mM)-treated P. luridum incubated in the light with TNBT. Note the heavy depositions in the interthylakoidal region (arrows).


1
2
3
4
0.5u
5
0.5u
6
0.5u


7
8
0.5um
9
10
11
0.5um

REFERENCES

1. Biggins, J. 1967. Photosynthetic reactions by lysed protoplasts and particle preparations from the blue-green alga, Phormidium luridum. Plant Physiol. 43:1447-1456.

2. Bisalputra, T., D. L. Brown, and T. E. Weier. 1960. Possible respiratory sites in a blue-green alga Nostoc sphaericum as demonstrated by potassium tellurite and tetranitro-blue tetrazolium reduction. J. Ultra. Res. 27:182-197.

3. Burnham, J. C., T. Stetak, and G. Locher. 1976. Extracellular lysis of the blue-green algae Phormidium luridum by Bdellovibrio bacteriovorus. J. Phycol. 12:306-313.

4. Carr, N. G. 1973. Metabolic control and autotrophic physiology. In Algal Physiology and Biochemistry. (N. G. Carr and B. A. Whitton, ed.). Bot. Mono., Vol. 9, pp. 39-65. Univ. Calif. Press. Berkeley, Calif.

5. Fahimi, H. D. 1969. Cytochemical bcalization of peroxidatic activity of catalase in rat hepatic microbodies (peroxisomes). J. Cell. Biol. 43:275-288.

6. Govindjee, and B. Z. Braun. 1974. Light absorption, emission and photosynthesis. In Algal Physiology and Biochemistry. (W. D. P. Stewart, ed). Bot. Mono., Vol. 10, pp. 346-390. Univ. Calif. Press. Berkeley, Calif.

7. Granhall, V., and B. Berg. 1972. Antimicrobial effects of bdellovibrio on blue-green algae. Arch. Mikrobiol. 84:234-242.

8. Hader, Donat-P. 1975. The effect of inhibitors on the electron flow triggering photo-phobic reactions in cyanophyceae. Arch. Microbiol. 103:169-174.

9. Hanker, J. S., W. A. Anderson, and F. E. Bloom. 1972. Osmiophilic polymer generation: Catalysis by transition metal compounds in ultrastructural cytochemistry. Science. 175:991-993.

10. Haydon, G. B., S. O. Smith, and A. M. Seligman. 1968. Observations on mitochondrial staining for succinic dehydrogenase. J. Histochem. Cutochem. 15:752-754.

11. Holin-Hansen, O. 1968. Ecology, physiology, and biochemistry of blue-green algae. Ann. Rev. Microbiol. 22:47-70.

12. Hsu, Y. C., D. S. Feingold. 1972. Locus of divalent cation inhibition of the bacteriological action of polymyxin B. Antimicrob. Ag. Chemother. 2:331-35.

13. Hsu Chen, C. C. and D. S. Feingold. 1973. Polyene antibiotic action on lecithin liposomes: Effect of cholesterol and fatty acid bath. Biochem. Res. Comm. 51:972-978.

14. Kessler, E. 1973. Hydrogenase,photoreduction and anaerobic growth. In Algal Physiology and Biochemistry. (N. G. Carr and B. A. Whitton, ed.). Bot. Mono. Vol. 9, pp. 456-473. Univ. Calif. Press, Berkeley, Calif.

15. Krogmann, D. W. 1973. Photosynthetic reactions and components of thylakoids. In The Biology of Blue-Green Algae. (N. G. Carr, and B. A. Whitton, ed.). Bot. Mono. Vol. 9, pp. 80-98. Univ. Calif. Press, Berkeley, Calif.

16. Leene, W., and W. van Iterson. 1965. Tetranitro-blue tetrazolium red reduction in Proteus vulgaris. J. Cell. Biol. 27:241-247.

17. Lloyd, D. 1974. Dark respiration. In Algae Physiology and Biochemistry. (W. D. P. Stewart, ed.). Bot. Mono. Vol. 10. P. 502-529. Univ. Calif. Press, Berkeley, Calif.

18. Macrobbie, E. A. C. 1974. Ion uptake. In Algae Physiology and Biochemistry (W. D. P. Stewart, ed.). Bot. Mono. Vol. 10. P. 676-713. Univ. Calif. Press, Berkeley, Calif.

19. Marchesi, V. T. 1968. Problems in the localization of membrane-bound enzymes by electron microscopic cytochemistry. In Biological Properties of the Mammalian Surface Membrane. (L. A. Manson, ed.). No. 8, p. 39-51. The Wistar Inst.

20. Nir, I., and A. M. Seligman. 1970. Photooxidation of diaminobenizidine (DAB) by chloroplast lamellae. J. Cell. Biol. 46:617-620.

21. Sedar, A. Q. and R. M. Burke. 1965. Localization of the succinic dehydrogenase system in Escherichia coli using combined techniques of cytochemistry and electron microscopy. J. Cell Biol. 24:285-295.

22. Seligman, A. M., J. J. Karnonsky, H. L. Wasserburg, and J. S. Hanker. 1968. Nondroplet ultrastructural demonstration of cytochrome oxidase activity with a polymerizing osmiophilic reagent, diaminobenzidine (DAB). J. Cell. Biol. 38:1-14.

23. Sun, D. C. and J. C. Burnham, 1977. A comparison of in vitro and in situ ferricyanide reduction in whole cells and spheroplasts of Phormidium luridum var. olivacea. Submitted to Can. J. Microbiol.

24. Seligman, A. M., I. Nir, and R. E. Plapinger. 1971. An osmiophilic ditetrazolium salt (DS-NBT) for ultrastructural dehydrogenase activity. J. of Histochem. Cytochem. 19:273-285.

25. Tolbert, N. W. 1974. Photorespiration. In Algal Physiology and Biochemistry. (W. D. P. Stewart, ed.). Bot. Mono. Vol. 10. pp. 474-504. Univ. Calif. Press, Berkeley, Calif.

26. Wildman, R. B., and C. C. Bowen. 1974. Phycobilisomes in blue-green algae. J. Bacteriol. 117:866-881.

SELECTED WATER RESOURCES ABSTRACTS
INPUT TRANSACTION FORM

1. Report No. | 2. | 3. Accession No. W

4. Title
Bacterial Control of Aquatic Algal Populations

5. Report Date
6.
8. Performing Organization Report No.

7. Author(s)
Jeffrey C. Burnham, Ph.D.

9. Organization
MEDICAL COLLEGE OF OHIO
Caller Service No. 10008
Toledo, Ohio 43699

10. Project No.
B-051-OHIO

11. Contract/Grant No.

12. Sponsoring Organization

13. Type of Report and Period Covered

15. Supplementary Notes

16. Abstract

This report describes the effect of a toxic substance secreted by Bdellovibrio bacteriovorus ATCC15143 on two species of blue-green algae, Phormidium luridum var. olivacea and Synechococcus sp. This algal inhibitor, which had a molecular weight of less than 10,000 and was partially sensitive to protease activity, was examined for its potential as a naturally-produced algal control agent. The need for a moderate concentration of environmental protein for production of the toxin was demonstrated along with the requirement of alkaline conditions (pH 9.0 optimum) for antialgal activity. One of the primary findings was the unique photorespiratory process that is triggered by exposure of the blue-green algae to the toxin. This photorespiration was a totally light dependent process as dark respiration remained normal and occurred prior to the lysis of the algal cells. When the effects of the bdellovibrio produced inhibitor were compared with the action of a variety of known chemical redox inhibitors, the greatest similarity was to a mixture of amphotericin B and polymyxin B. Transmission electron microscopy indicated a distinct pathological breakdown of the algal cells that was dependent on the blue-green algal species being exposed to the toxin. A host independent mutant of B. bacteriovorus 15143 was isolated which produced a similar algal toxin. Finally, over 100 bacterial isolates from local water resources were evaluated for comparative blue-green algal lytic activity.

17a. Descriptors

*Algal control, aquatic algae, *cyanophyta, electron microscopy, oxygen requirement, photosynthetic oxygen, *toxin, water quality control.

17b. Identifiers

*Bdellovibrio bacteriovorus, cell lysis, *photorespiration, Phormidium luridum, Synechococcus

17c. COWRR Field & Group 05G

18. Availability | 19. Security Class. (Report) | 20. Security Class. (Page) | 21. No. of Pages | 22. Price

Send To:
WATER RESOURCES SCIENTIFIC INFORMATION CENTER
U.S. DEPARTMENT OF THE INTERIOR
WASHINGTON, D.C. 20240

Abstractor Jeffrey C. Burnham, Ph.D. | Institution Medical College of Ohio, Toledo, Ohio